

MOLECULAR ECOLOGY AND DIVERSITY OF METHANOGENIC ARCHAEA
FROM NORTHERN PEATLANDS

A Dissertation

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by

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Peatlands are abundant in northern latitudes, containing nearly 30% of total soil carbon, and represent the most important natural source of atmospheric CH₄. However, the methanogenic *Archaea* inhabiting these ecosystems have been scarcely studied. We characterized the 16S rRNA gene sequence diversity of *Archaea* in two NY State bogs, McLean (MB) and Chicago (CB), and in Michigan Hollow (MH), a minerotrophic fen. At all sites, we found a diverse community of mainly novel uncultivated archaeal groups. At both bogs, a group in the *Methanomicrobiales* we named E2 was dominant, making up 50-99% of total *Archaea* as indicated by terminal restriction fragment length polymorphism (T-RFLP) analysis and quantitative PCR. At MH, E1, a group related to E2, and *Methanosaetaceae* phylotypes were co-dominant and exhibited considerable diversity. We also examined archaeal communities in the rhizoplane of two contrasting vascular plants (*Dulichium spp.* and *Sarracenia purpurea*) in MB, and found nearly even distributions of *Methanosarcinaceae*, Rice cluster I and E2 phylotypes, in contrast with methanogenic communities in bulk peat.

We used a methanogen-specific T-RFLP assay to direct the isolation of a novel genus in the previously-uncultured E1 group from MH fen, tentatively named. *Methanosphaerula palustris* strain E1-9c. Additionally, we isolated three potentially novel species in the genus *Methanobacterium* from other temperate and boreal peatlands.

Complementarily, we assessed sequence microdiversity of the E2 group in MB and CB and observed greater microdiversity in MB compared to the more recently-formed CB. Simulations with an individual-based mathematical model indicated that the differences in the ecological development at each site may serve as a theoretical scenario to explain the microdiversity patterns. In addition, using T-RFLP analysis, we examined the methanoarchaeal community macrodiversity at 22 sites from North America and Europe. At temperate sites, an ecological pattern was suggested by the dominance of E2 in acidic bogs while E1 and *Methanosaetaceae* were dominant in minerotrophic fens. In more northern boreal peatlands, *Methanosarcinaceae*, Rice cluster-II, and *Methanobacteriales* represented important fractions of the community not observed in temperate sites.

These observations provide an initial understanding of methanoarchaeal communities useful in predicting effects of global climate change on peatland methanogenesis.

BIOGRAPHICAL SKETCH

Hinsby Cadillo-Quiroz was born in the town of Magdalena, Cajamarca Peru on the 31st of May, 1977. Early in his life, Hinsby moved from Magdalena to Huarney in the central Peruvian seashore, the town where his relatives have live for generations. He was raised among fishing weekends, school days, the so often “Fiesta” or town celebrations and a big extended family. Along primary and high school, he always showed an avid interest for biology and other sciences, wining several science contests. He attended the “Inca Garcilazo de la Vega” high school and graduate as the best student of his class receiving the “Honor and Excellence” award. In 1994 at age 16, he was admitted to the “Universidad Nacional Mayor de San Marcos”, in Lima, to pursue his undergraduate degree in the school of biological sciences. College was a great time personally and professionally, and also sealed his wishes to be a scientist trying to understand microorganisms in natural environments. After graduation, he researched the use of viral indicators in recreational beaches, and later enrolled in a master degree program on Zoology, Ecology and Evolution in the graduate school of his alma matter on the year 2000. Along that time he worked with Professor Walter Cabrera-Febolla as a member of the theoretical ecology group. In the last semester of his mater degree, Hinsby was selected as a Fulbright fellow for graduate studies and moved to Cornell University for a PhD in Microbiology on 2002. He joined the group of Professor Stephen Zinder and since then enjoyed his time visiting and studying peatlands from northern America and Europe. Upon completion of his degree in January 2008, Hinsby will work as a postdoctoral fellow in the laboratory of Dr. Rachel Whitaker at University of Illinois in Urbana-Champaign, IL.

Francisco and Yolanda gave me the best gift of my life: “opportunity”, the rest was left up to my friends and me. This work is dedicated to all of them that I left behind and also dream of opportunity as I do.

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I owe a great deal of gratitude to the many people who made my time at Cornell a pleasant, enriching, fulfilling, and joyful experience. In retrospect, joining the Stephen H. Zinder group was the best decision I could have made when I came to Cornell. Steve has taught me how to be a better scientist, and equally importantly, how to be a better mentor and person. Steve's style is certainly well recognized and acknowledged by all his students and colleagues, and I hope to someday be able to carry on with his legacy with some of his style. I am indebted to Steve for giving me the opportunity to pursue all my, though sometimes too broad, scientific interests and for allowing me to recruit and supervise the many undergrads that worked with me.

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TABLE OF CONTENTS

BIOGRAPHICAL SKETCH.....	iii
DEDICATION.....	iv
ACKNOWLEDGEMENTS.....	v
LIST OF FIGURES	xi
LIST OF TABLES	xiv
CHAPTER ONE: Peatlands: ecological characteristics and methanogenic processes	
1.1 Definitions and characteristics of peatlands	1
1.2 Peatlands and global warming	4
1.3 Bogs and fens: from ombrotrophic to minerotrophic conditions and in between.....	5
1.4 Methanogenesis in peatlands.....	7
1.5 Methanogens in peatlands.....	9
1.6 Research objectives.....	11
References.....	13
CHAPTER TWO: Vertical profiles of methanogenesis and methanogens in two contrasting acidic peatlands in central New York State, USA	
2.1 Abstract.....	22
2.2 Introduction.....	23
2.3 Methods.....	24
2.4 Results.....	30
2.5 Discussion.....	41
2.7 Acknowledgements.....	45
References.....	46
CHAPTER THREE: Archaeal community in a minerotrophic fen and T-RFLP directed isolation of a novel hydrogenotrophic methanogen	
3.1 Abstract.....	52
3.2 Introduction.....	53
3.3 Methods.....	55
3.4 Results.....	60
3.5 Discussion.....	71
3.6 Acknowledgements.....	80
References.....	81

CHAPTER FOUR: <i>Methanosphaerula palustris</i> gen. nov., sp. nov., a hydrogenotrophic methanogen isolated from a minerotrophic fen peatland	
4.1 Abstract.....	89
4.2 Introduction.....	90
4.3 Results and discussion.....	95
4.4 Acknowledgements.....	105
References.....	106
CHAPTER FIVE: Modeling of environmental influences over gene sequences to assess microdiversity patterns of archaeal populations	
5.1 Abstract.....	111
5.2 Introduction.....	112
5.3 Methods.....	115
5.4 Results and Discussion.....	119
5.5 Acknowledgements.....	129
References.....	130
CHAPTER SIX: Composition of euryarchaeal communities in bogs and fens from temperate and boreal peatlands	
6.1 Abstract.....	135
6.2 Introduction.....	136
6.3 Methods.....	137
6.4 Results and Discussion.....	140
6.5 Acknowledgements.....	148
References.....	149
APPENDIX A: Molecular diversity of archaea inhabiting the rhizoplane of two vascular plants from an acidic bog	
A.1 Abstract.....	153
A.2 Introduction.....	154
A.3 Methods.....	155
A.4 Results.....	160
A.4 Discussion.....	167
A.5 Acknowledgements.....	172
References.....	173
APPENDIX B: Novel <i>Methanobacterium</i> strains from northern temperate and boreal peatlands	
B.1 Abstract.....	178
B.1 Introduction.....	179
B.2 Methods.....	180
B.3 Results and Discussion.....	183
B.4 Acknowledgements.....	190

References.....	191
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LIST OF FIGURES

Figure 1-1. Latitudinal distribution of totals wetland area	3
Figure 1-2. Estimated (fitted line) latitudinal variation of atmospheric CH ₄ emission from wetlands	3
Figure 2-1. Time course for CH ₄ production by peat slurries from Chicago Bog and McLean Bog vertical profiles	32
Figure 2-2. Phylogenetic tree for archaeal 16S rRNA gene clones from McLean and Chicago bog.....	34
Figure 2-3. Representative T-RFLP electropherograms of archaeal 16S rRNA gene fragments.....	36
Figure 2-4. Figure 2-4. Normalized T-RFLP profiles of 16S rRNA gene fragments amplified from peat samples from vertical profile of Chicago Bog (CB) and McLean Bog (MB).....	37
Figure 2-5. Figure 2-5. Real time qPCR quantification of 16S rRNA gene copy number of total <i>Archaea</i> (A) and the E2-E1 group (B).....	40
Figure 3-1. Phylogenetic analysis of archaeal 16S rRNA gene clones from Michigan Hollow fen.....	61
Figure 3-2. Clone distribution from libraries constructed with different primer combinations.....	62
Figure 3-3. Rarefaction analysis of Michigan Hollow fen clone libraries.....	63
Figure 3-4. CH ₄ production by peat slurries from Michigan Hollow fen.....	64
Figure 3-5. T-RFLP analysis of peat samples and enrichments using the 1Af-1100r primer set and HhaI-Sau96 restriction enzymes.....	65
Figure 3-6. Microscopic examination of E1-9c cells.....	68
Figure 3-7. Physiological characterization of E1-9c.....	70
Figure 3-8. Rarefaction analysis of Michigan Hollow fen and other peatland clone libraries.....	72
Figure 4-1. Transmission electron microscopy of E1-9c ^T cells.....	96

Figure 4-2. Na effect on E1-9c ^T 's growth. Total Na includes background Na in media plus additions from NaCl.....	98
Figure 4-3. E1-9c ^T 's formate utilization tests.....	99
Figure 4-4. Phylogenetic analysis of strain E1-9c ^T and members of the order <i>Methanomicrobiales</i> based on 16S rRNA gene (A) and Mcr-A (B) amino acid sequences.....	101
Figure 5-1. Equation for the logistic population growth without incorporating the competition between individuals (A), and incorporating competition among varying individuals within the population (B).....	117
Figure 5-2. 16S rRNA gene phylogenetic tree (A) and clone distribution (B) of “E2/ <i>Methanoregula</i> ” populations in McLean and Chicago Bog.....	120
Figure 5-3. IBM simulation with variance of $K_{(x)} = 1$ and variance of $C = 0.04$	123
Figure 5-4. IBM simulation results with variance of $K_{(x)} = 1$, and variance of $C = 0.4$	123
Figure 5-5. IBM simulation for 5500 generations in MB.....	125
Figure 5-6. IBM simulation for 5500 generations in CB.....	126
Figure 6-1. Representative T-RFLP profiles after standardization.....	142
Figure 6-2. Summarized community structure of methanogenic <i>Archaea</i> , and closely related groups, from peatlands in North America and Europe.....	146
Figure A-1. <i>Dulichium spp.</i> (three-way sedge) (A) rhizome-stolon-based root system and <i>Sarracenia purpurea</i> (pitcher plant) (B) vertical root morphology and their linear correlation between root length and root surface area.....	157
Figure A-2. Phylogenetic tree of euryarchaeal 16S rRNA gene sequences....	162
Figure A-3. Phylogenetic tree of crenarchaeal 16S rRNA gene sequences.....	163
Figure A-4. Clone distribution of combined 16S rRNA gene libraries from <i>Dulichium</i> and <i>Sarracenia</i> rhizoplane samples.....	163
Figure A-5. Archaeal groups and their clone number as detected by each primer set used in this study.....	164

Figure A-6. Rarefaction analysis at 97 and 99% similarity levels for 16S rRNA gene sequences from combined <i>Dulichium</i> and <i>Sarracenia</i> samples for each primer set.	165
Figure A-7. A) Representative T-RFLP traces of 16S rRNA and principal component analysis (PCA).	166
Figure B-1. Negative stain electron microscopy of strains MH-SWAN1, SP6, AL-21 and FIN-120.	184
Figure B-2. Effects of pH on CH ₄ production of strains MH-SWAN1, SP6, AL-21 and FIN-120.	185
Figure B-3. Effects of NaCl on CH ₄ production of strains MH-SWAN1, SP6, AL-21 and FIN-120.	186
Figure B-4. Effects of Na ₂ S on CH ₄ production of strains MH-SWAN1, SP6, AL-21 and FIN-120.	187
Figure B-5. 16S rRNA gene sequence phylogenetic analysis of strains MH-SWAN1, SP6, AL-21,	189

LIST OF TABLES

Table 1-1. Differential characteristics of bogs versus fens.....	6
Table 2-1. Main physicochemical characteristics of peat along the vertical profiles from Chicago and McLean Bog.....	30
Table 2-2. Predicted terminal restriction fragment (T-RF) length of archaeal 16S rRNA gene sequences from clone libraries and their phylogenetic affiliation.	35
Table 2-3. Specificity and coverage of 16S rRNA gene primers used to quantify total <i>Archaea</i> and the <i>Methanomicrobiaceae</i> E2-E1 group.....	39
Table 3-1. Intergenic Transcribed Sequence (ITS) size and tRNA presence in Euryarchaeal phylotypes and associated cultured methanogens.....	74
Table 4-1. Physiological characteristics and comparison of strain E1-9c ^T with other representative coccoid and close phylogenetic relative methanogens belonging to the order <i>Methanomicrobiales</i>	103
Table 6-1. Sampling sites, location and major characteristics.....	140
Table A-1. 16S rRNA gene primers used in this study.....	158
Table B-1. Physiological characteristics of novel strains versus other <i>Methanobacterium</i> species.....	190

CHAPTER I

“The differential response of bogs and fens to climate change would complicate efforts to predict changes in carbon dynamics in peatlands”

Updegraph, K. 2001.

“Given the diversity of possible responses by boreal and sub arctic peatlands to climatic warming, it is impossible at present to predict their future contributions to the global carbon cycle”.

Gorham, E. 1991

PEATLANDS: ECOLOGICAL CHARACTERISTICS AND METHANOGENIC PROCESSES

1.1 DEFINITIONS AND CHARACTERISTICS OF PEATLANDS

Peatlands are a type of wetland where the accumulation of organic matter exceeds the rates of decomposition and where organic matter has accumulated in a layer at least a 30-40 cm thick containing an organic substrate known as peat (Blodau, 2002, Glaser, 1987, Gore, 1983, Martini et al., 2007).

The terminology and classification of peatlands is complex and reflects the use of several non-unified criteria for their classification (Gore, 1983). Mire and peatland are the main terms used to describe these ecosystems, and are used synonymously (Martini et al., 2007). In terms of peatland classification, bogs (poor ombrotrophic), fens (rich minerotrophic) and intermediates forms, such as poor fens, are the more commonly accepted groupings (Blodau, 2002, Bridgham et al., 1996, Glaser, 1987, Gore, 1983, Keller et al., 2006, Kellogg & Bridgham, 2003, Wahlen, 1993); however,

swamps (minerotrophic forested peat accumulating sites with 25% of the area covered by trees) and marshes (minerotrophic wetlands with variable water table) have been suggested as additional types of peatlands (Martini, 2007).

In addition to exhibiting different physicochemical characteristics, peatlands are ecosystems structured both horizontally and vertically (Blodau, 2002). Vertical gradients from old to young form as a result of peat accumulation through time; the lower layer known as the “catotelm” is a saturated zone depleted of oxygen, where organic matter is anaerobically decomposed with decreasing rates as recalcitrant material accumulates in deeper sections. On top of the catotelm, a commonly oxic surface layer is present and it is also known as the “acrotelm”. Oxic or anoxic conditions and physical characteristics of the acrotelm vary seasonally, and it is commonly made up of a productive layer, litter layer and collapsed layer that contain most of the plants' macrostructures (Brown, 1998, Warner, 1996). Horizontally, peatlands present different surface formations at different scales. Hummocks, hollows, lawns, or pools are common examples of microtopographic formations, and their conditions have been suggested to influence other biological components, such as the methanogenic microbial community (Galand et al., 2003). At a broader horizontal scale, peatlands can form complexes which contain many individual peatlands with different characteristics and with limits not easily discriminated. These complexes commonly achieve extensive dimensions in the order of hundreds or thousands of square kilometers (Blodau, 2002).

Peatlands cover around 5 million Km² globally, contain around one third of the total terrestrial carbon (Gorham, 1991), and 10% of the global freshwater (Tarnocai & Stolbovoy, 2007). These ecosystems are distributed worldwide (Gore, 1983), but the major concentration of peatlands occurs in northern latitudes along the northern temperate, boreal, subarctic and arctic regions from North America and Eurasia

(Gorham, 1991). The concentration of peatlands in northern latitudes has a strong imprint in the latitudinal distribution of the area covered by wetlands in the world (Wang et al., 1996) as shown in Figure 1-1.

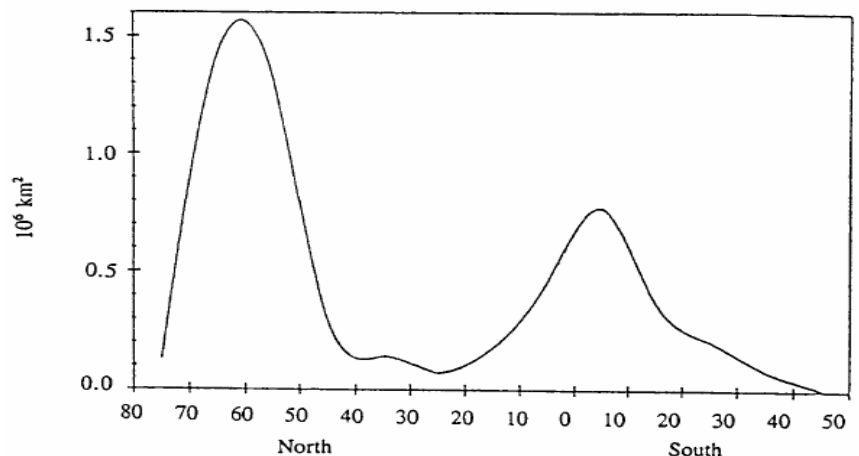


Figure 1-1. The latitudinal distribution of totals wetland area (Wang et al., 1996)

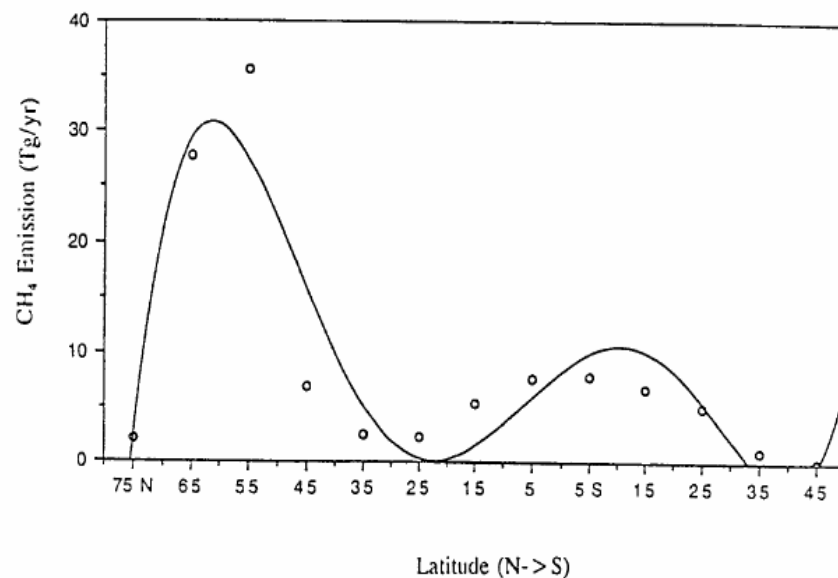


Figure 1-2. Estimated (fitted line) latitudinal variation of atmospheric CH₄ emission from wetlands (Wang et al., 1996).

There are approximately 455 Pg of soil carbon stored in peatlands, and most of it is concentrated in a small 3% of terrestrial land area in northern latitudes (Gorham, 1991). The development of peatlands can lower the atmospheric concentration of CO₂, and at the same time increase the release of CH₄ to the atmosphere, as observed in

atmospheric records (Blodau, 2002). Interestingly, the distribution of atmospheric CH₄ emissions closely resembles the latitudinal distribution of wetlands with the highest concentrations in the northern latitudes which contains the highest peatland abundance as shown in Figures 1-1 and 1-2.

1.2 PEATLANDS AND GLOBAL WARMING

Atmospheric CH₄ has more than doubled in the last 200 years and nearly 20% of the total estimated annual CH₄ emissions originate from peatlands (Cicerone & Oremland, 1988, Harriss et al., 1985). Although recent calculations suggest that peatlands' CO₂ sequestration and CH₄ emissions nearly balance each other out in their "global warming potential" (Roulet, 2000), the ongoing temperature increase in northern latitudes is expected to change this balance (Hansen et al., 2006). Northern latitudes are predicted to exhibit the largest changes in surface temperature and precipitation (Bridgham et al., 1995, Roulet et al., 1992, Updegraff et al., 2001). These changes are currently observed by the movement of vegetation cover expanding poleward (Hansen et al., 2006) and the accelerating rate of permafrost melting (Stokstad, 2004). The increase of vegetation cover is expected to exhibit positive feedback increasing temperatures (Hansen et al., 2006), while permafrost melting would expose an immobilized pool of organic matter to higher rates of decomposition and CO₂ release. In fact, wetter conditions in the tundra peatlands are linked to the current release of more CH₄ in Sweden (Stokstad, 2004). There is not yet a clear understanding of warming effects or feedbacks on key processes such as soil carbon decomposition's sensitivity to temperature (Davidson & Janssens, 2006), or effects on methanogenic pathways and rates (Metje & Frenzel, 2005c), particularly in high latitude cold peatlands (e.g. boreal and subarctic). **An understanding of these processes and the participating organisms in temperate peatlands would provide**

a foundation for predictions about potential changes in high latitude peatlands, as temperatures increase and create conditions similar to those observed in temperate sites.

1.3 BOGS AND FENS: FROM OMBROTROPHIC TO MINEROTROPHIC CONDITIONS AND IN BETWEEN

The main difference between bogs and fens lies in their water supplies' origins (hydrology) and chemistry (Gore, 1983). Bogs are rain-fed or ombrotrophic sites, and as such, they receive only atmospheric inputs of water with low cations and nutrients (Aerts et al., 1992, Maimor et al., 1992). The availability of cations and nutrients in bogs is reduced even more by the dominant *Sphagnum* mosses (Crum, 1992). *Sphagnum* mosses efficiently intercept nutrients from the atmosphere, leachates, and litter (Vanbreemen, 1995), and have a high cation exchange capacity associated with their polyuronic acid content (Clymo, 1963). The low cation concentration and influence of *Sphagnum* renders these sites highly acidic ($\text{pH} < 4$). Bogs commonly have low plant diversity, which has been associated with acidity, competition for cations and nutrients, low soil temperature, drought stress, and excess water (Vanbreemen, 1995).

On the other hand, fens receive inputs of groundwater and runoff from surrounding uplands (Martini et al., 2007), with their water mineral content being influenced by soil or rocks the water passes through before it reaches the peatland surface. Fens have a greater plant diversity (Vitt & Chee, 1990), are commonly dominated by sedges and woody plants, exhibit higher alkalinity and pH (5.5–7 in moderate rich fens; >7 in extremely rich fens) (Crum, 1992, Johnson, 1985, Wheeler & Giller, 1982), and their pore water has a higher content of minerals, such as Ca, Mg,

Si, and Na, and lower amounts of total P or PO_4^{3-} , NH_3 or NO_3^- and K than bogs (Dettling et al., 2007, Schwintzer & Tomberlin, 1982).

Bogs and fens can be considered as endpoints of a spectrum of conditions as summarized in Table 1-1. Intermediate forms can have mixed characteristics, as in the case of poor fens (Glaser et al., 1990).

Table 1-1. Differential characteristics of bogs versus fens

	Bog	Fen
Water source	rainwater	surface & groundwater
Water mobility	stagnant	flowing
Peat depth	up to 6 or 12m	up to 2m
pH	acidic	neutral
Cations	scarce	abundant
Nutrients	low	high
Source of nutrients	atmosphere/rain	groundwater

Peatlands often have readily-apparent gradients of plant species distributions, biogeochemistry, and hydrology across several spatial scales (Bridgham et al., 1996, Okland et al., 2001). For example, acidity-alkalinity or ombrotrophic-minerotrophic gradients are commonly observed within single peatlands or peatland complexes (Bragazza et al., 2005, Glaser et al., 1990) or among independent sites (Keller & Bridgham, 2007).

Many studies have measured mineral and nutrient concentrations, and mineralization rates in bogs versus fens or along the gradients between them with different results (Bragazza & Gerdol, 2002, Kellogg & Bridgham, 2003, Schwintzer & Tomberlin, 1982). **However, to this point, the effects of the conditions in bogs and fens on the microbial community diversity and structure are not yet clear. Microbial activity exclusively determines the decomposition rates of organic matter and emissions of CO_2 and CH_4 .**

1.4 METHANOGENESIS IN PEATLANDS

CH₄ production is the terminal step in the carbon flow of many anaerobic habitats, including peatlands. Under anaerobic conditions, the decomposition of organic matter is carried out by a complex food web of decomposers, in which the end product of one functional group becomes the substrate of another (Westermann, 1996). Methanogenic *Archaea* are a highly catabolically specialized group, and the only group responsible for CH₄ production. Despite their broad phylogenetic diversity and distribution in different habitats, methanogens metabolize a very narrow range of substrates (Zinder, 1993). A major consequence of this specialization is that methanogens are dependent on other microbial groups for their substrates. Thus, in general, CH₄ production in peatlands depends directly on microbial activity, but also indirectly on some environmental conditions such as vegetation type, peat chemistry, soil temperature, and the position of redox boundaries associated with the water table (Blodau, 2002). Environmental conditions could affect the methanogenic *Archaea* and other microbial guilds that regulate degradation of organic matter (Weston & Joye, 2005), and therefore, the input of substrates for methanogenesis. This needs to be kept in mind as a confounding factor when interpreting ecological data from the field or *in vitro* peat experiments.

In freshwater systems, CH₄ is mainly formed either from acetate dissimilation (acetoclastic pathway) or CO₂ reduction (hydrogenotrophic pathway). The most widespread catabolic reaction carried out by methanogens is the reduction of CO₂ to CH₄ using H₂ as a reductant (Garcia et al., 2000), and H₂ is a major fermentation product in many species of anaerobic bacteria, fungi and protozoa (Zinder, 1994). However, methanogenesis from acetate has been suggested as the major pathway for methanogenesis in wetlands (Vasander & Kettunen, 2006). Acetate is the ultimate end

product of many fermentative pathways (Zinder, 1994), and it has been observed to accumulate in some northern peatlands (Duddleston et al., 2002).

The relative contribution of the two methanogenic pathways in peatlands is not clear. However, some patterns have been observed, especially when characteristics such as the oligotrophic to minerotrophic gradient occurring from bog to fen are considered. In sites with high plant productivity (fens), which contain more available fresh organic matter, the acetate pathway dominates, while in more oligotrophic sites containing plant material with lower decomposition rates (bogs), the hydrogenotrophic pathway becomes more important (Bellisario et al., 1999, Chasar et al., 2000, Galand et al., 2005, Popp et al., 1999, Strom et al., 2003). The same trend is seen vertically within a site as the acetate pathway is favored in the shallow subsurface peat, while the hydrogenotrophic one becomes more dominant in older, less reactive deeper peat (Hornibrook et al., 1997). Methanogenic pathways differ in temperature dependence in *in vitro* experiments: hydrogenotrophic contribution increased as temperature increased, while at lower temperatures the acetoclastic contribution became more important (Avery et al., 1999, Fey & Conrad, 2000). However, the response to temperature substantially decreased under low substrate availability (Bergman et al., 1998, Dunfield et al., 1993, Valentine et al., 1994), which could help to better explain the high contribution of hydrogenotrophic methanogenesis observed even in cold boreal bogs (Metje & Frenzel, 2005b).

Methanogens are strict anaerobes, and therefore anaerobic conditions are required for methanogenesis. However, methanogenesis has been observed in surface peat, which suggests that although anaerobic conditions are required, water table oscillations, and therefore oxic-anoxic periods, do not inhibit methanogens and instead lag periods occur (Kettunen et al., 1999).

Finally, in terms of pH influence over methanogenesis, some field studies have shown that there is no correlation of CH₄ production rates with pH values in peatlands (Moore et al., 1994). This independence has been suggested to be based on the adaptations of methanogenic populations to acidity (Updegraff et al., 1995).

1.5 METHANOGENS IN PEATLANDS

Initial attempts to characterize the methanogens inhabiting peatlands relied on culturing efforts with limited success (Goodwin & Zeikus, 1987, Williams & Crawford, 1985). Hydrogenotrophic methanogens, such as *Methanobacterium* and *Methanospirillum*, and acetoclastic methanogens such as *Methanosarcina* have been isolated from northern peatlands (Kotsyurbenko et al., 2007, Rivkina et al., 2007, Simankova et al., 2003, Williams & Crawford, 1985). However, the development of molecular based methods such as PCR cloning and sequencing, community profiling, probing, etc have shown the existence of a broader diversity of *Euryarchaea* with many uncultured groups (Basiliko et al., 2003, Grobkopf et al., 1998a, Hales et al., 1996). Commonly, these uncultured groups represent the dominant fraction in molecular analysis. Molecular surveys have been done mainly in bogs and were restricted to a few sites (Galand, 2004). In peatlands, sequences associated with *Methanomicrobiales* and *Methanosarcinales* were found in almost all surveyed sites, while *Methanobacteriales* were commonly but not always present (Basiliko et al., 2003, Galand et al., 2003, Kotsyurbenko et al., 2004, Metje & Frenzel, 2005a). *Methanococcales* were only detected by probe hybridization in two European sites (McDonald, 1999, Upton et al., 2000), although sequences of this group have not been found in clone libraries from peatlands.

In bogs and poor fens, sequences of a group initially called “R10” (Edwards et al., 1998, Hales et al., 1996) or also named “Fen cluster” (Galand et al., 2002, Galand

et al., 2003) or group “E” (Basiliko et al., 2003) have been commonly found as the dominant *Euryarchaea*. This group forms an independent cluster within the *Methanomicrobiales* order (Edwards et al., 1998), and this order is made up of only hydrogenotrophic methanogens (Garcia, 1990). The hydrogenotrophic nature of the group “R10/Fen cluster/E” was supported by enrichment cultures (Bräuer et al., 2006b, Sizova et al., 2003), and recently confirmed by an isolate obtained from a upstate NY acidic bog (Bräuer et al., 2006a). The isolate was proposed as a new genus: “*Candidatus Methanoregula boonei*”, and presents the most acidic pH optimum for methanogenesis (Bräuer et al., 2006a). The acidic optimum pH and hydrogenotrophic nature of this isolate are in agreement with the dominant hydrogenotrophic methanogenesis, the dominance of “R10/Fen cluster/E” sequences in bogs, and the expectations of acidic-specialized populations of methanogens. On the other hand, few studies have been done in minerotrophic fens, where hydrogenotrophic methanogenesis could also be a significant contributor in the addition to acetoclastic methanogenesis (Galand et al., 2005). Minerotrophic fens can have a greater global impact on CH₄ emissions than bogs in terms of CH₄ equivalents per unit of area (Smemo & Yavitt, 2006) in the near future, and thus the methanogens inhabiting these systems deserve more attention.

Additionally, other uncultured groups such as “Rice Cluster (RC)-I” and “RC-II” have been observed in bogs and poor fens. Both groups form independent phylogenetic branches from the known methanogenic orders, and recently an isolate has been obtained for the RC-I group (Sakai et al., 2007). The isolate has been proposed as the type strain of a new methanogenic order: “*Methanomonales*” (S. Sakai, personal communication). The RC-I group is abundant in rice paddies making up for 20 to 50% of total methanogens (Kruger et al., 2005). The group has been suggested to be well adapted to low H₂ habitats (Sakai et al., 2007) and relatively oxic

conditions (Erkel et al., 2005) such as the rice rhizosphere where RC-I phylotypes have been commonly found (Grobkopf et al., 1998b, Lu et al., 2005). RC-I could play a role in the rhizosphere of sedges and other vascular plants in peatlands. RC-II was initially detected in rhizosphere of rice (Grobkopf et al., 1998c) and no enrichment or isolated cultures have been obtained for this group yet.

Peatlands are far from homogeneous systems. Ombrotrophic to minerotrophic, acidic to neutral pH, temporarily to permanently anoxic, and *Sphagnum* to sedge-dominated sites, are contrasting conditions with continuous gradients commonly seen within or among sites. These gradients together with regional weather (temperate-boreal-artic), geographical (basins, mountain, seashore, inland, etc.) and ecological succession conditions could have different effects on the methanogenic community composition and diversity.

1.6 RESEARCH OBJECTIVES

Environmental variability translates into ecological opportunity for metabolic and genetic diversification. Thus it is reasonable to expect the existence of novel euryarchaeal groups, new clusters within known groups, different fine scale phylogenetic variability, and environmental or geographical effects on community composition among methanogens in peatlands.

The general research goal was to characterize the diversity and ecology of methanogens inhabiting contrasting northern peatlands such as fens and bogs. To achieve this goal, the molecular diversity and structure of the archaeal community among contrasting bogs (Chapter II), or fen (Chapter III), or different compartments within a bog (Appendix A), were assessed using 16S rRNA gene-based molecular tools. Subsequently, the archaeal community of a minerotrophic fen was characterized

with the goal of using the molecular survey data to direct culturing efforts and obtain a representative of a novel methanogenic group significant in fens (Chapter III). A novel hydrogenotrophic methanogen was isolated and characterized, and proposed as new genus “*Methanosphaerula palustris*” strain E1-9c in the *Methanomicrobiales* order (Chapter IV). To evaluate the geographical distribution and importance, in temperate and boreal bogs and fens, of sequences related to the group E2 (including “*Candidatus Methanoregula*”), group E1 (including *Methanosphaerula*), and other methanogenic groups, samples from several bog and fen sites from the US, Canada, Finland, Sweden, Norway, and Austria, were evaluated using a methanogen-specific community profiling molecular tool (Chapter VI). Complementary, fine-scale phylogenetic diversity of the dominant methanogens in two bogs with contrasting developmental stories was assessed and a mathematical model for neutrally evolving gene sequences was constructed as a means to assess hypothetical explanations of the observed diversity patterns (Chapter V). Finally, novel *Methanobacterium* species were isolated from boreal and temperate peatlands (Appendix B), and evaluated their physiologically and phylogenetically diversity.

In summary, the contributions of this research expand the knowledge about the archaeal community composition in bogs and fens in a broad geographical range. It provides a clear rationale for coupling molecular data with culturing efforts. In addition, the physiological and phylogenetic information obtained from the novel isolate E1-9c will allow a better interpretation of the ecological role of the members of the E1 group in fens. In the future, information about the genome of the strain E1-9c will be available (currently being sequenced by Joint Genome Institute-JGI) for comparison with other methanogens from peatlands (such as “*Candidatus Methanoregula boonei*”) or different sources, and the assessment of adaptations to the peatlands ecosystems.

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CHAPTER II

**VERTICAL PROFILES OF METHANOGENESIS AND METHANOGENS IN
TWO CONTRASTING ACIDIC PEATLANDS IN CENTRAL NEW YORK
STATE, USA.¹**

2.1 ABSTRACT

Northern acidic peatlands are important sources of atmospheric CH₄, yet the methanogens in them are poorly characterized. We examined methanogenic activities and methanogen populations at different depths in two peatlands, McLean bog (MB) and Chicago bog (CB). Both have acidic (pH 3.5 – 4.5) peat soils, but the pH of the deeper layers of CB is near-neutral, reflecting its previous existence as a neutral-pH fen. Acetotrophic and hydrogenotrophic methanogenesis could be stimulated in upper samples from both bogs, and phylotypes of methanogens using H₂/CO₂ (*Methanomicrobiales*) or acetate (*Methanosarcinales*) were identified in 16S rRNA gene clone libraries and by T-RFLP analyses using a novel primer/restriction enzyme set that we developed. Particularly dominant in the upper layers was a clade in the *Methanomicrobiales*, called E2 here and the R10 or Fen group elsewhere, estimated by quantitative PCR to be present at ~10⁸ cells per gram dry peat. Methanogenic activity was considerably lower in deeper samples from both bogs. The methanogen populations detected by T-RFLP in deeper portions of MB were mainly E2 and the uncultured euryarchaeal rice cluster (RC)-II group, whereas populations in the less acidic CB deep layers were considerably different, and included a *Methanomicrobiales* clade we call E1-E1', as well as RC-I, RC-II, marine benthic group D (MBD), and a new cluster that we call the subaqueous cluster (SC). E2 was

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barely detectable in the deeper samples from CB, further evidence for the associations of most organisms in this group with acidic habitats.

2.2 INTRODUCTION

Although peatlands in northern latitudes account for 5 to 10% of the CH₄ emitted into the Earth's atmosphere (Whalen, 2005), little is known about the composition and abundance of the microorganisms responsible for CH₄ production in these systems. A better understanding of these methanogens is needed to predict how emissions of atmospheric CH₄ from northern peatlands will respond to global environmental changes (Brown, 1998; Wieder and Yavitt, 1994).

Northern peatlands include a wide range of ecosystems, each with a characteristic peat soil derived from partially decaying plant material and with little or no rock-derived minerals. At one end are the most acidic systems, called bogs, characterized by low mineral nutrient concentrations and dominated by *Sphagnum* mosses and a few ericaceous shrub species (Crum, 1992). Increasing pH is associated with less *Sphagnum* and dominance by *Carex* sedges and graminoid plants, resulting in near-neutral pH systems, called fens (Crum, 1992). A characteristic of many northern peatlands is that the plant species composition of surface vegetation can be quite different from the remains of plants in the peat that dominated in the past (Barber, 1981)

Numerous methanogens have been cultivated and characterized from diverse habitats (Garcia, 1990), but initial attempts at cultivation of indigenous methanogens from bogs were unsuccessful (Goodwin and Zeikus, 1987; Williams and Crawford, 1985). However, the application of molecular techniques based on 16S rRNA and *mcrA* (encoding a subunit of the methylreductase involved in methanogenesis) gene

sequences has revealed a diversity of methanogens as well as related *Euryarchaeota* in bogs (Basiliko et al., 2003; Galand et al., 2005; Hales et al., 1996; Kotsyurbenko et al., 2004). At this point in time, none of the sequences in acidic peat bogs match those of isolated methanogens, although related sequences have recently been detected in enrichment cultures (Horn et al., 2003; Sizova et al., 2003).

In this study, we examined the depth (vertical) distribution of methanogenic activity and methanogenic *Archaea* in two northern peatlands in New York State. One, known locally as ‘Chicago Bog’ has acidic bog plants growing over what was once a neutral pH fen so that lower layers are less acidic. The other site, known locally as ‘McLean Bog’, was acidic throughout the profile. We demonstrate here that T-RFLP analyses of methanogen populations of all samples from McLean Bog showed dominance of a clade in the *Methanomicrobiales* associated with acidic habitats, whereas methanogen populations in lower, more neutral, samples from Chicago Bog differed considerably from the acidic upper layers.

2.3 METHODS

Study sites and sampling

McLean bog (MB) and Chicago bog (CB) are located within 40 km of Ithaca NY (42° 30' N, 76° 30' W) in the northeast USA, and both sites were initially described by Osvald (1970). MB is an ombrotrophic kettle hole bog approximately 70 m across (0.04 km²), the peat is 8 m deep, and the vegetation is dominated by *Sphagnum* moss (*S. angustifolium* and *S. magellanicum*). Vascular plants including ericaceous shrubs (*Chamaedaphne calyculata*, and *Vaccinium corymbosum*), three-way sedge (*Dolichium spp*), and pitcher plants (*Sarracenia purpurea*) are also present in significant numbers. CB is an oligotrophic floating bog of approximately 0.05 km²,

and is associated with a pond on its east side. Its current surface is sphagnum dominated, and its earlier stages of peat development were sedge derived (Dettling, 2005). The *Sphagnum* species covering this bog (e.g. *S. fuscum*, *S. magellenicum*) are responsible for its hummocky surface; and the drier hummocks support ericaceous shrubs (*C. calyculata*) which are dominant in the site.

Sampling was done June 28 in CB and October 4 for MB, in 2004. Duplicate cores were taken at both sites using a PVC coring device with airtight seals. Sample points along vertical profile were determined by considering the physical characteristics of the peat in a parallel core obtained with a Russian style peat corer device. Airtight sealed cores were immediately transported to the laboratory and sampled inside an anaerobic glove box (Coy Laboratory Products, USA) with an atmosphere of N₂ and 2-4% H₂. Peat samples from each of the two cores taken from each site were individually assayed. Since the replicate cores had similar results, only those from one core from each site are presented.

CH₄ production

Peat slurry incubations with or without substrate additions were performed as described by Bräuer et al (2004). Briefly, inside an anaerobic glove box, 1 g of peat was added to anaerobic deionized water to a volume of 10 mL in 18 x 150 mm crimp-top tubes. The tubes were sealed with butyl rubber stoppers and were flushed with sterile O₂-scrubbed N₂/CO₂ (70%/30%, Mixed Gas Industries, USA). Anaerobic stock solutions were added as indicated to the following final concentrations: sodium acetate (1 mM), rifampicin (10 mg L⁻¹). Sterile O₂-scrubbed H₂/CO₂ (80%/20%, 0.7 atm, Mixed Gas Industries, USA) was added to appropriate tubes. Incubations were performed at room temperature (ca. 23°C) in the dark, under static conditions for samples with no addition or 1 mM acetate, and shaking (225 RPM) for samples with

rifampicin, or rifampicin + H₂/CO₂. Presented data represent the averages of triplicate samples.

Chemical analyses

Headspace gas was analyzed for CH₄ using a Perkin Elmer 3920B gas chromatography column with a flame ionization detector (Phoenix Equipment Inc., USA). Peat slurries were vortexed for 30 s prior to headspace analysis. Dry weight and pH of triplicate peat samples were determined as described elsewhere (Bräuer et al., 2004) within 24 hour after sampling.

DNA extraction and PCR amplification

Triplicate samples from each depth in the peat core were extracted with the Power Soil™ DNA kit (MoBio, USA) using the manufacturer's protocol with some slight modifications. Briefly, 0.5 g of peat was mixed with beads and disrupting solution, and after adding the C1 solution we also added 50 µL of a sterile 200 mM AlNH₄(SO₄)₂ solution to avoid humic acid co-purification (Braid et al., 2003). The mixture was subjected to 1 min, 20 s of bead beating at maximum speed (MiniBeadbeater™, Biospect Products, USA). The quality of recovered DNA solution was examined by 2% agarose gel electrophoresis and spectrophotometer readings at 230 and 260 nm; minimal DNA shearing and A_{260/230} ratios close to 2 were observed in almost all samples.

A fragment from positions 1 to 1100 (*E. coli* numbering) of the 16S rRNA gene was amplified using the archaeal-specific 1AF (5'-TCY GKT TGA TCC YGS CRG AG-3')-1100R (5'-TGG GTC TCG CTC GTT G-3') set of primers (Hales et al., 1996). The PCR mixture contained the following components at its reactants concentrations per µL: 1x Taq buffer with 1.5 mM MgCl₂ (Eppendorf, USA), 0.2 mM deoxy-nucleotide triphosphates (dNTP), 0.25 µM forward and reverse primers, 1.2 U

of *Taq* Polymerase (Eppendorf, USA), 0.2 µg bovine serum albumin (BSA) and 0.1-0.3 ng of extracted DNA. The PCR conditions were as described by Hales et al. (1996) with 25 amplification cycles. Amplification products were examined by electrophoresis on 1% agarose gels for size verification.

Cloning, sequencing and phylogenetic analysis

16S rRNA gene clone libraries were constructed as described by Basiliko et al. (2003). Six clone libraries were constructed for samples from 15 cm, from 40 and 65 cm depth in CB. Using the TA Cloning kit® (Invitrogen, USA) and m13 primer screening, 60 clones per library were selected for restriction analysis with *Hae*III and *Hha*I enzymes (New England Biolabs, USA). Clones displaying unique restriction patterns were sequenced with an ABI 3730 automated sequencer (Bio Resources Center- BRC, Cornell University). Sequences were compared against the GenBank database (Benson et al., 2004) in order to ensure that newly-reported relatives were included in our database.

Phylogenetic analyses of the sequences were performed using the ARB software (Ludwig et al. 2004) with the latest 16S rRNA sequences database release (released 08.2003; <http://www.arb-home.de>) and an “archaeal database” with 2500 complete and partial archaeal sequences (Jurgens, 2002). Phylogenetic placement was done using the quartet PUZZLE method implemented in ARB in comparison to reference archaeal sequences. A nucleotide base frequency filter that included positions with more than 50% invariance (1020 valid columns) was used to avoid possible treeing artifacts. Sequences were added to the tree reference sequence tree using the ARB parsimony tool without altering global tree topology. Tree topology was confirmed using maximum likelihood, neighbor joining, and FITCH methods (as implemented in the ARB package).

The sequences of the 16S rRNA gene clones obtained in this study have been deposited in the GenBank nucleotide sequence database under the following accession numbers: DQ301878 to DQ301915.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

We used the PCR primers and conditions described above. The 1100R reverse primer was fluorescently labeled on its 5' end with Carboxyfluorescein (5'-/6-FAM). T-RFLP analysis was done as described elsewhere (Marsh, 1999) with some modifications. Briefly, 30 μ L triplicate PCR reactions per sample were pooled and purified with the Quick StepTM2 PCR Purification Kit (Edge Biosystems, USA), and 70 ng were digested with a mix of HhaI (15U) and Sau96I (10U) enzymes (New England Biolabs, USA) for 3 h at 37°C. Digested DNA was purified with the Performa[®] DTRV3 96-Well Short Plate Kit (Edge Biosystems, USA). Purified products were concentrated in a vacuum centrifuge, and then resuspended with a mix of Hi Di-Formamide (Applied Biosystems, USA) and Gene Scan 500-Liz marker (12 μ L/mL; Applied Biosystems, USA). Fragments were resolved with an Applied BioSystems 3730 DNA Analyzer (BRC, Cornell University).

T-RF sequence length, peak height, and area were determined using the GeneScan Analysis Software (Applied Biosystems, 2000). GeneScan's results containing peak size, height and area were exported for profile standardization of each sample. Using a Java-based routine, the relative fluorescent units (RFU) of peaks with size 50 to 500 bases were added by height or area and normalized to 100 total RFU units. The standardization step minimized peak height and area variation from sample to sample and did not affect the profiles' morphologies. Standardized profiles from samples taken in triplicate at each depth were averaged by their correspondent peak heights, and standard deviations were calculated.

Real-time quantitative PCR

Primer selection for total *Archaea*, and primer design for group E2-E1, was done considering previously described optimal conditions (Nadkarni et al., 2002; Suzuki et al., 2000) and primer efficiency and coverage. For total *Archaea*, the Arch 967 F primer -5'-AAT TGG CGG GGG AGC AC-3' (Amann et al., 1990; Riley-Buckley, 2001) combined with ArchH-1060R – the reverse complement of A1040F- (Reysenbach and Pace, 1995) had a broad euryarchaeal and crenarchaeal coverage with no matches for bacterial or eukaryotic sequences as reviewed for A1040F (Baker et al., 2003). For group E2-E1 joint quantification, we used the A-gE372 (5'-ACT CCG AGT GCC CGT WAA ATC-3') and A-gE540aR (5'-AGT AAT AGT GGC CAC CAC TCG AGC-3') set of primers, designed in this study with the primer-design and probe-match tool of ARB software. Predicted coverage and specificity analyses used the latest ARB database, the “Archaeal database” (Jurgens, 2002) and Ribosomal Database Project II (Cole et al., 2003), confirming that all the archaeal phylotypes retrieved from our study sites could be amplified by the archaeal primers and that the E1-E2 primers only amplified members from this group (data not shown). Both primers were challenged with unspecific and specific targets as shown in Table 2-3.

Primer concentration was optimized using iTaq SYBR Green Supermix with ROX (BioRad, USA). 30 μ L PCR reactions contained 1X Supermix, 0.25 and 0.2 μ M forward and reverse primers (for total *Archaea* and E2-E1 respectively), 0.1 μ g μ L⁻¹ BSA, and 3 μ L of extracted DNA. Amplifications were performed on duplicate samples at two dilutions (1:5 and 1:10) using an ABI Prism® 7000 Sequence Detector (Applied Biosystems, USA) with the following settings: 2 min at 50°C, 10 m 95°C, 15 s at 95 °C with 1 m at 60°C for 38 cycles. This was followed by a dissociation protocol to check for proper dissociation profiles, otherwise reactions

were rejected. Plasmid DNA external standards with 10^8 to 10^2 target copies per μl were constructed for quantification as described by Fey et al. (2004).

Real time PCR results were normalized to percent peat dry weight at each depth and by the arbitrary value of two rRNA gene operons per genome, the closest integer to the currently reported archaeal average in the Ribosomal RNA Operon Copy Number Database (Kaplenbach et al., 2001).

2.4 RESULTS

Physicochemical characteristics of vertical profiles of peat cores

Stratigraphy of the cores from both sites indicated a 10-cm cap of light brown colored sphagnum peat underlain by a more degraded softer in consistency, dark brown peat at 15-20 cm. At depths below 40 cm, the peat was highly decomposed and darker in color indicating highly reduced conditions. The peat in deeper layers (40 and 65 cm depth) of Chicago bog (CB) had decreasing amounts of *Sphagnum* remains and was herbaceously derived, i.e. mostly *Carex* rhizomes, whereas deeper layers in McLean bog (MB) consisted mostly of *Sphagnum* remains. The pH in deeper layers of MB remained low, but in CB it increased to 4.9 and 5.7 respectively at 40 and 65 cm depths (Table 2-1). The dry matter content was greater at lower depths in both cores, with a greater increase in the CB core.

Table 2-1. Main physicochemical characteristics of peat along the vertical profiles from Chicago and McLean Bog. Peat samples were taken at the indicated depths; mean \pm SD; n=3.

Depth (cm)	pH		% dry weight	
	Chicago bog	McLean bog	Chicago bog	McLean bog
10	3.92 \pm 0.07	3.58 \pm 0.42	5.42 \pm 0.09	4.22 \pm 1.08
15/20	4.06 \pm 0.06	4.03 \pm 0.06	5.25 \pm 0.06	7.88 \pm 0.21
40	4.87 \pm 0.13	4.19 \pm 0.02	12.12 \pm 0.11	9.12 \pm 0.4
65	5.71 \pm 0.12	4.21 \pm 0.04	11.91 \pm 0.21	7.5 \pm 0.23

CH₄ production by core samples

Rates of CH₄ production from endogenous substrates in samples from the 40 cm depth were less than 15% of those in the 15-20 cm depth (Fig. 2-1), and even slower rates occurred in samples from 65 cm depth (data not presented). Added acetate had no effect on rates of CH₄ production until ca. 20 days of incubation when CH₄ production in the acetate-amended samples from upper layers of MB and the 15 cm layer of CB (Fig. 2-1A, B) increased relative to unamended samples. Added acetate did not stimulate CH₄ production in the 10 cm layer of CB or in the 40 cm (Fig. 2-1C) and 65 cm samples from either bog during the entire 63 day incubation time.

A previous report at the same study sites showed that the build up of acetic acid inhibited CH₄ production in peat amended with H₂/CO₂, and that the addition of rifampicin avoided this interference by acetogenic organisms (Bräuer et al., 2004). CH₄ production in upper layers by samples amended with H₂/CO₂ and rifampicin was significantly greater than the rifampicin-only controls.

Rates of methanogenesis increased during the first days of incubation for both sites (Fig. 2-1A, B), indicative of methanogen growth. In deeper layers, the CH₄ production in H₂/CO₂-amended samples started to increase above the un-amended control only after more than 20-30 days of incubation (Fig. 2-1C). Samples from 65 cm presented similar, although slower, response compared to those from 40 cm (not shown).

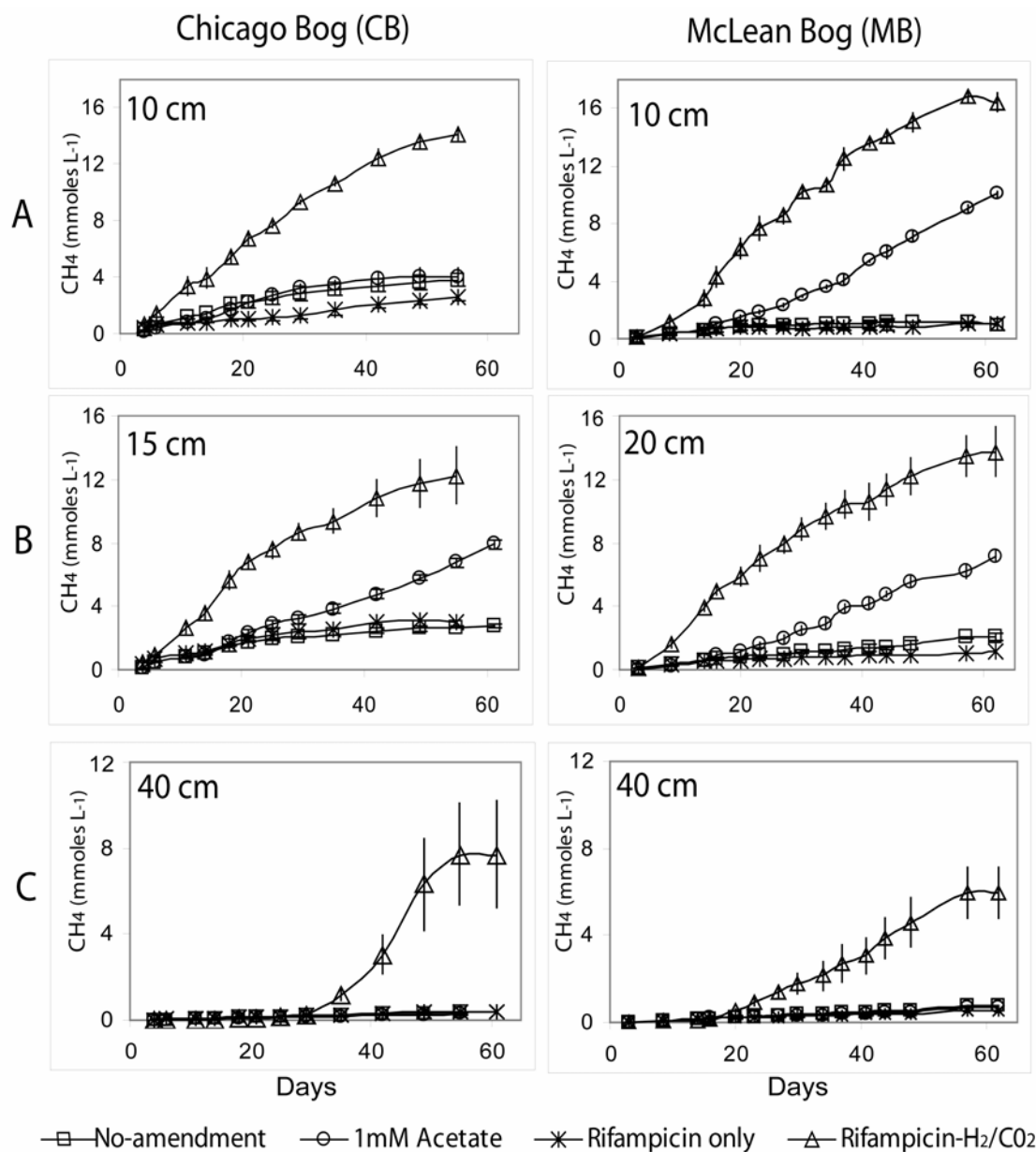


Figure 2-1. Time course for CH₄ production by peat slurries from Chicago Bog and McLean Bog vertical profiles. A: 10 cm deep, B: 15/20 cm deep and C: 40 cm deep incubated at ca. 23 °C; mean± SD; n=3.

16S rRNA phylogenetic diversity

In order to study methanogen community composition we used cloning and sequence analyses of the 16S rRNA gene. We used the 1AF-1100R set of primers

(Hales et al., 1996) because our initial studies on clone libraries developed with different archaeal primer sets indicated a good coverage of methanogenic *Archaea* in our study sites (H. Cadillo-Quiroz, unpublished). Comparison of reported sequences for shallow layers of MB (Basiliko et al., 2003) and sequences from our clone libraries for CB shallow layers, showed a similar phylogenetic composition of methanogenic archaeal groups (Fig. 2- 2).

Sequences were associated with *Methanosarcinaceae* and *Methanosaetaceae* families, and the *Methanomicrobiales*. Uncultured groups such as Rice Cluster I and II were present in both bogs. Clones with phylotypes associated with MBD (Marine Benthic group D) (Vetriani et al., 1999) were recovered only from the deeper layers of CB. Additionally, we observed a cluster of sequences that were not positioned within the other previously described groups. Those sequences formed a distinct cluster related mainly to environmental phylotypes retrieved from immersed or subaqueous environments like lake sediments, Siberian deep wells, deep-sea chimneys, and a nearby neutral fen (J.Yavitt, unpublished). This cluster of sequences, that we have named the Subaqueous Cluster (SC), represents a new group of uncharacterized euryarchaeota.

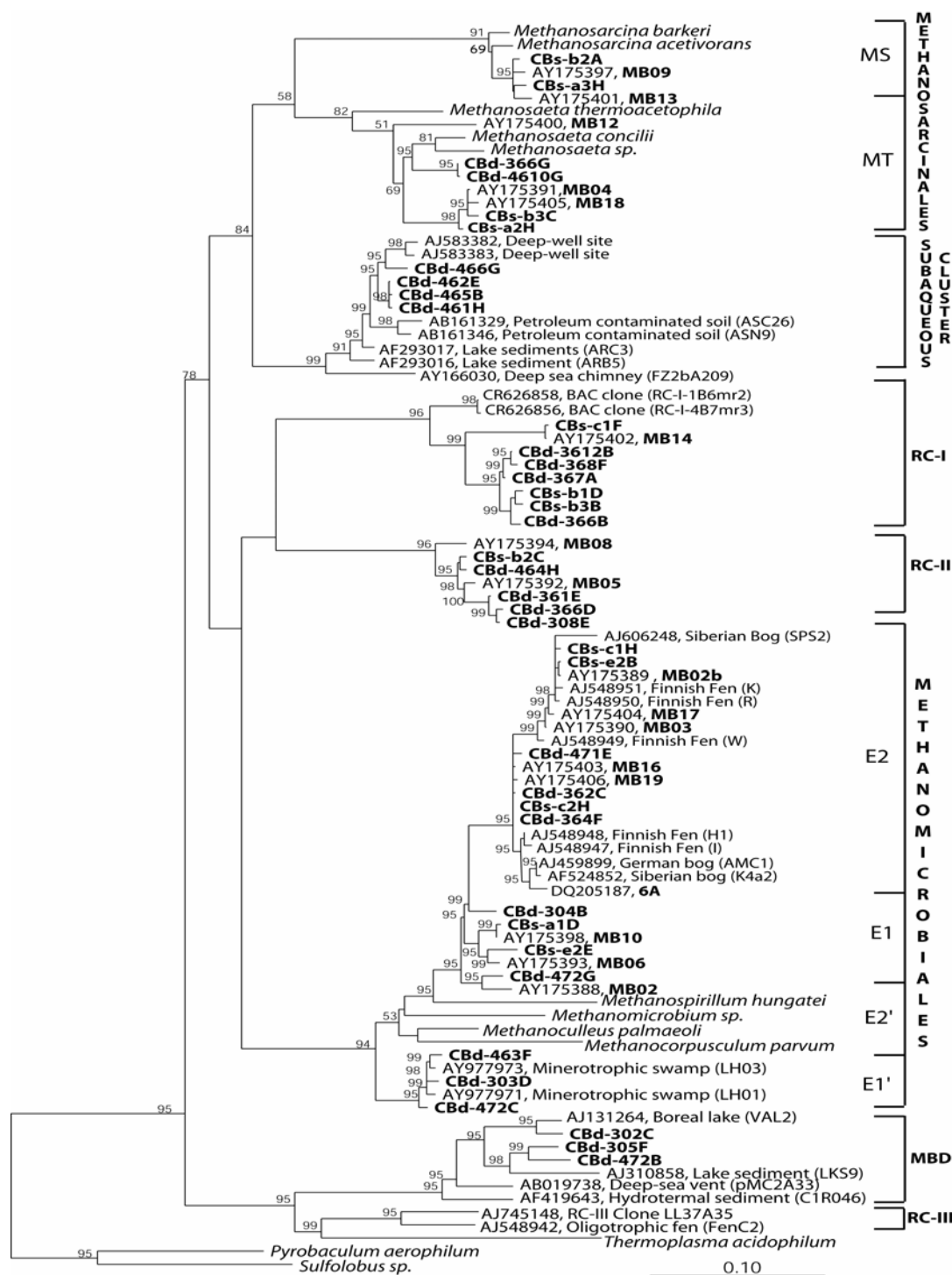


Figure 2-2. Phylogenetic tree for archaeal 16S rRNA gene clones from McLean and Chicago bog. *Methanosarcinales*: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT); *Subaqueous cluster* (SC); *Rice cluster-I* (RC-I); *Rice cluster-II* (RC-II); *Methanomicrobiales*: Group E1, Group E1', Group E2 and Group E2'; *Marine Benthic group D* (MBD). Euryarchaeal and crenarchaeal nearly complete 16S rRNA gene sequences were used to construct tree with the neighbor joining and quartet

PUZZLE methods. Tree topology was confirmed with maximum likelihood algorithm, and bootstrap values (100 trees) greater than 50 are indicated. GenBank accession numbers of added sequences are indicated. Clones from Chicago Bog shallow and deeper layers are indicated by the initials CBs and CBd respectively.

T-RFLP analysis of community structure

We used terminal restriction fragment length polymorphism (T-RFLP) analysis to obtain a description of the dominant groups in the archaeal community accessible through our primer set. The 1Af-1100R set of primers have not been used previously in T-RFLP analysis, and in this study we defined T-RFLP parameters that minimized ambiguous identification. A T-RFLP protocol using two restriction enzymes (HhaI and Sau96I) was designed by performing multiple *in silico* sequence analyses of published sequences and our clone libraries, and confirming the predicted fragment size by amplifying and T-RFLP analyzing clones. Table 2-2 presents the identity match and predicted terminal restriction fragment (T-RF) size from the *in silico* digestion of our sequences, and Figure 2-3 shows two representative profiles and their corresponding peak identifications.

Table 2-2. Predicted terminal restriction fragment (T-RF) length of archaeal 16S rRNA gene sequences from clone libraries and their phylogenetic affiliation. Fragment size is predicted from 3' end for fluorescently labeled reverse primer 1100R (Hales et al., 1996).

Phylogenetic association	T-RF length (bp)	Clone name
<i>Methanosarcinaceae</i>	271	MB13, MB09, CBs-a3H, CBs-b2A
<i>Methanosaetaceae</i>	88	MB04, MB18, CBs-a2H, CBs-b3C
	118	CBd-4610G, CBd-366G
	397	MB12
Subaqueous cluster	363	CBd-466G, CBd-461H, CBd-462E, CBd-465B
Rice cluster-I	230	MB14, CBs-b1D, CBs-b3B, CBs-c1F
	363	CBd-367A, CBd-368F, CBd-3612B, CBd-366B
Rice cluster-II	100	MB05, MB08, CBs-b2C, CBd-366D, CBd-464H, CBd-308E, CBd-361E
<i>Methanomicrobiales</i> group E2	233	MB16, MB19, MB02b, MB 17, MB03, CBs-e2B, CBs-c2H, CBs-c1H, CBd-364F, CBd-471E, CBd-362C, 6A8
<i>Methanomicrobiales</i> group E1	292	MB06, MB10, CBs-e2E, CBs-a1D, CBd-304B, CBd-472G
<i>Methanomicrobiales</i> group E1'	292	LH 01, LH03, CBd-463F, CBd-472C, CBd-303D
Marine benthic group D	337	CBd-472B, CBd-305F, CBd-302C

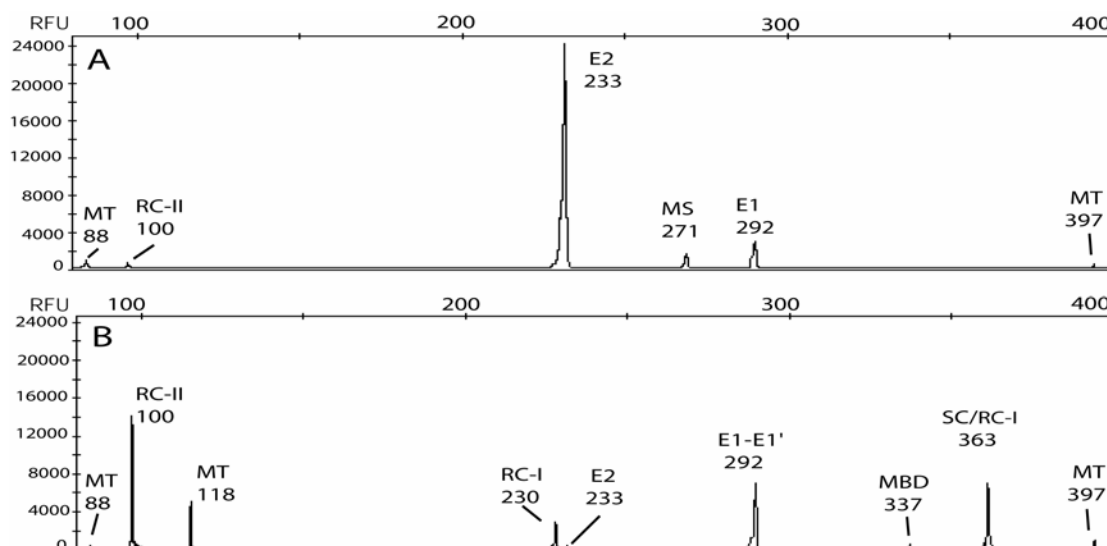


Figure 2-3. Representative T-RFLP electropherograms of archaeal 16S rRNA gene fragments amplified with the 1AF-1100R (Hales, et al., 1996) from DNA extracts from peat samples from McLean and Chicago Bogs. A: McLean Bog 20 cm deep and B: Chicago Bog 40 cm deep. The x-axis and the numbers above the peaks show the length (bp) of the terminal restriction fragments (T-RFs). The archaeal groups represented by distinctive T-RFs are: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), Rice Cluster-I (RC-I), Rice Cluster-II (RC-II), Marine benthic group D (MBD), and *Methanomicrobiales*: Group E1 (E1), Group -E1' (E1'), Group E2 (E2). The y-axis shows the intensity of the peaks in relative fluorescence units (RFU).

We were able to resolve the groups A-F described by Basiliko et al. (2003) and in many cases obtain better resolution. For example, Group B, equivalent to the *Methanosaetaceae*, could be resolved into three groups, and group E, equivalent to the *Methanomicrobiales*, could be resolved into two groups E1 and E2, which were not entirely monophyletic and were subdivided into E1, E1', E2, and E2' (Fig. 2-2). E1' was only found in clone libraries of deeper layers of CB, and E2' sequences were not recovered in the libraries and correspond to cultured *Methanomicrobiales* (Fig. 2-2). Of other groups, the SC cluster shared the same T-RF size with a subcluster of RC-I. According to our clone libraries, both would only occur in samples from deeper layers of CB.

Assessment of the vertical community structure in duplicate core samples from both study sites is summarized in Figure 2-4.

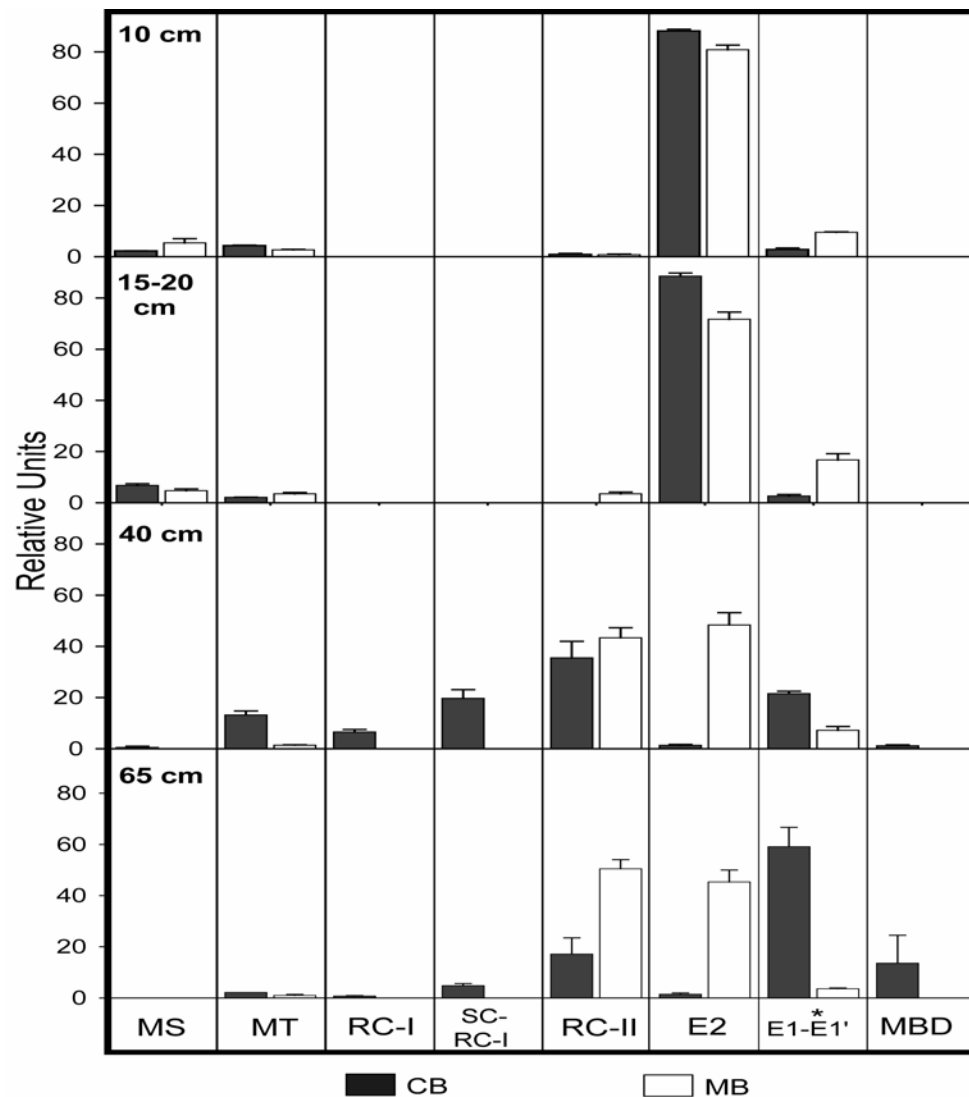


Figure 2-4. Normalized T-RFLP profiles of 16S rRNA gene fragments amplified from peat samples from vertical profile of Chicago Bog (CB) and McLean Bog (MB). Sampling depths are indicated in each set of profiles. Peaks matching its predicted group were arranged in the same order as in their phylogenetic tree, and the *Methanosaetaceae* peaks were merged into a single group for simplicity. The archaeal groups represented by distinctive T-RFs are: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), Rice Cluster-I (RC-I), Rice Cluster-II (RC-II), Marine benthic group D (MBD), and *Methanomicrobiales*: Group E1 (E1), Group -E1' (E1'), Group E2 (E2). Profiles were normalized to a total of 100 units. Mean \pm SD; n=3. *: E1' sequences were only observed in CB at 40 cm and 65 cm.

The community profiles from both sites were similar at 10 and 15-20 cm. The dominant T-RF in these layers was that of group *Methanomicrobiales* E2, in agreement with its high abundance in clone libraries (data not shown). Group *Methanomicrobiales* E1 was present as a smaller fraction and was greater in MB than in CB. *Methanosarcinaceae*, *Methanosaetaceae* and RC-II T-RFs were present in upper layers at both sites as a very small fraction of the community. The community structure at 40 and 65 cm depth clearly changed compared to the upper layers (Fig. 2-4), but the differences not only corresponded with depth but also between sites. CB harbored a greater number of T-RF types than MB, where MBD, RC-I and SC were consistently observed at deep layers of CB only. Group E2 decreased in dominance at both sites. However, E2 changed from being the most dominant T-RF to a very small fraction of the community (close to the technique's limit of detection) in CB; while in MB, E2 was still one of the major components in the profiles. Group E1/E1' (Fig. 2-2) increased in CB, particularly at 65 cm where E1' sequences were mainly recovered, and slightly decreased in MB. In addition, a unique *Methanosaetaceae* T-RF was present only at lower depths of CB and it had a greater abundance than the others found in higher layers particularly at 40 cm. The increase of the RC-II fraction at 40 cm depth was the only common finding between the two sites at their deeper layers.

Abundance estimation by quantitative PCR

We attempted to complement the T-RFLP's qualitative information with a quantitative PCR (qPCR) approach focused on the abundance of total *Archaea*, and groups E2 and E1. Because of the limitations imposed by the close phylogenetic relationships of group E2 and E1, we could not design primers to quantify each group separately but instead developed a single primer set that quantifies both groups together but excludes E1' and E2' associated sequences.

For total archaeal quantification, we used universal primers that have been successfully used in quantitative PCR in other studies (Riley-Buckley, 2001). Both sets of primers were tested for specificity and coverage and the results are summarized in Table 2-3.

Table 2-3. Specificity and coverage of 16S rRNA gene primers used to quantify total *Archaea* and the *Methanomicrobiaceae* E2-E1 group.

a Bacterial isolates from MB classified by their closest phylogenetic association to bacterial groups based on their 16S rRNA gene sequence.

b chromosomal DNA from microorganisms cultures in PCR reaction was 0.5 ng μL^{-1} .

c Plasmid DNA for environmental clones was used in 10^6 copies μL^{-1} .

Group/genus	Organisms tested	Reaction with	
		Archaeal primers	E2-E1 primers
Bacteria ^{a,b}	<i>Bulkholderia</i> sp., <i>Chromobacterium</i> sp., <i>Clostridium</i> sp., <i>Yersinia</i> sp., <i>Azospirillum</i> sp., <i>Magnetospirillum</i> sp.	-	-
Archaea ^{b,c}			
<i>Thermoplasma</i>	<i>T. acidophilum</i>	+	-
<i>Methanococcus</i>	<i>M. maripaludis</i>	+	-
<i>Methanosarcina</i>	<i>M. siliciae</i> , <i>M. acetivorans</i> , <i>M. barkeri</i>	+	-
<i>Methanosaeta</i>	<i>M. thermoacetophila</i> , <i>M. concilli</i>	+	-
<i>Methanogenium</i>	<i>M. carici</i>	+	-
<i>Methanocorpusculum</i>	<i>M. parvum</i>	+	-
<i>Methanofollis</i>	<i>M. liminatants</i>	+	-
<i>Methanospirillum</i>	<i>M. hungatei</i>	+	-
E2 culture ^b	Methanogenic culture 6A	+	+
Environmental clones	CBs-c1H (group E2 clone)	+	+
	CBs-e2E (group E1 clone)	+	+
	CBs-b3C (<i>Methanosaeta</i> clone)	+	-

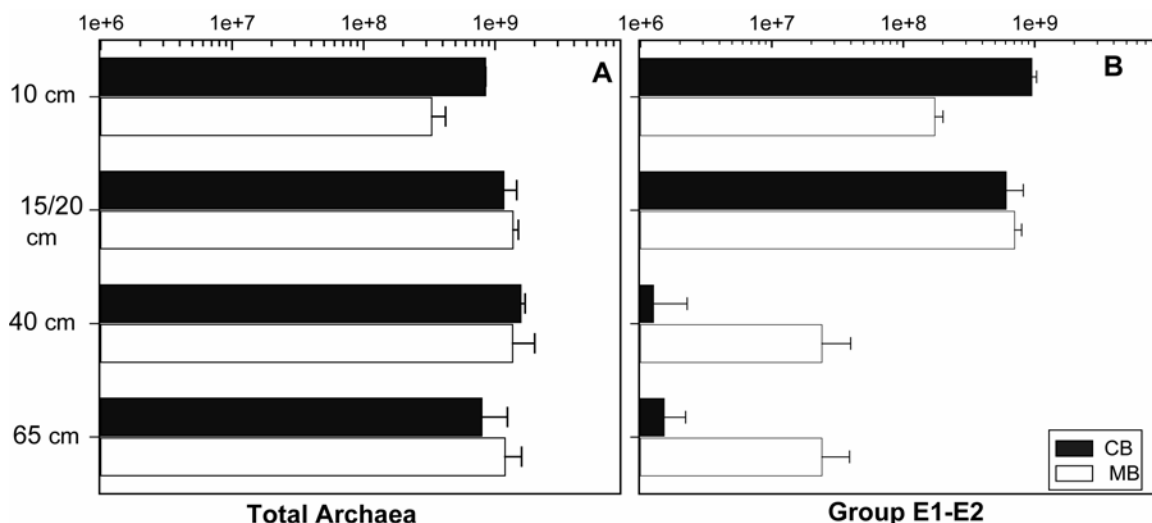


Figure 2-5. Real time qPCR quantification of 16S rRNA gene copy number of total *Archaea* (A) and the E2-E1 group (B) in DNA extracts from peat samples from the vertical profile of Chicago Bog (CB: black bars) and McLean Bog (MB: white bars). The x-axis represents the number of gene copies normalized by two rRNA operon copies and expressed in logarithmic scale; y-axis indicates the depth at which the sample was taken.

Results of qPCR (Fig. 2-5A), indicated that total archaeal numbers did not strongly decline with depth in both sites (10^8 - 10^9 normalized gene copy number per gram of dry peat). These results, although higher in order of magnitude since they are expressed per gram of dry instead of fresh peat, were similar to those previously obtained with fluorescent *in situ* hybridization (FISH) in a Siberian acidic peatland (Kotsyurbenko et al., 2004). Attempts to apply FISH to these peat samples were unsuccessful partly because of substantial autofluorescence of the peat material.

The quantification results for group E2-E1 (Fig. 2-5B) showed that E2 and E1 roughly constituted ca. 55% of total archaeal targets in the upper layers of both sites, with E2 as the main contributor as indicated by T-RFLP. E2-E1 target decreased to a different degree at 40 and 65 cm at both sites: in CB decreased by roughly 2.5 orders of magnitude ($\sim 0.15\%$ of total *Archaea*), while in MB it decreased by 1.3 orders of magnitude ($\sim 2\%$ of total *Archaea*).

2.5 DISCUSSION

McLean Bog (MB) and Chicago Bog (CB) are typical of acidic ombrotrophic (ombro- = rain fed; Crum, 1992) bogs with low mineral content (Blodau, 2002). Upper layer at both sites shared similar physicochemical characteristics, but their deeper layers exhibited significant differences, particularly pH (Table 2-1) and peat composition. In MB and CB, methanogenic activity in lower layers using endogenous substrates was 1-1.5 orders of magnitude lower and there was little if any initial stimulation by addition of methanogenic substrates, indicating that the methanogenic populations were at low levels and had low activity.

Hydrogenotrophic and acetoclastic, i.e. H_2/CO_2 and acetate based, methanogenesis are frequently observed in wetlands (Whalen, 2005). In acidic ombrotrophic bogs, the former has been commonly reported as the predominant process (Avery et al., 1999; 2003; Chasar et al., 2000; Duddleston et al., 2002; Galand et al., 2005; Horn et al., 2003; Hornibrook et al., 2000; Lansdown et al., 1992; Nakagawa et al., 2002; Williams and Crawford, 1984), although the latter has recently been shown as the main contributor in a pH 4.8 Siberian bog (Kotsyurbenko et al., 2004). Our incubation results showed that H_2/CO_2 -amended samples produced significantly more CH_4 than those amended with acetate, indicating a greater potential for this pathway. The most methanogenically active layers, the upper 20 cm, just below the water table level, had a minimal lag time and rates increased within a few days after the incubations begun (Fig. 2-1A, B). This supports the existence of active and rapidly responding hydrogenotrophic methanogen populations at upper layers. The long lags demonstrated by samples from lower layers incubated with H_2/CO_2 indicated that populations of hydrogenotrophic methanogens were low and that perhaps only a specific subpopulation could be stimulated by addition of substrate.

On the other hand, our results for potential aceticlastic methanogenesis showed stimulation after a 20 day lag period in both upper layers of MB and CB's 15 cm (Fig. 2-1). This indicates that the acetate-utilizing populations were near saturation for acetate, which we have measured as $<50 \mu\text{M}$ (S. Bräuer, unpublished) and are slow to respond to substrate addition. Although aceticlastic methanogens in MB can be stimulated with low doses of acetate (Bräuer et al., 2004; our results), and Kotsyurbenko et al (2004) demonstrated a case in which this pathway is responsible for twice as much methanogenesis as the hydrogenotrophic one, typical of neutral pH habitats; the contribution of aceticlastic methanogenesis in acidic northern peatlands is not yet clear.

The 16S rRNA gene clone library of Basiliko et al. (2003) for MB as well as those presented here for CB demonstrated the presence of members of the *Methanomicrobiales*, known to use H_2/CO_2 , as well as the *Methanosarcinaceae* and *Methanosaetaceae*, which are known to be aceticlastic. Additionally, other euryarchaeal groups such as RC-I (Rice Cluster I), RC-II, MBD and subaqueous cluster (SC) were observed at our sites. These RC-I and RC-II have been found in different ecosystems with active methanogenesis, such as acidic peatlands, rice paddies, and river or lake sediments (Chin et al., 1999; Galand et al., 2002; Glissman et al., 2004). The marine benthic group D (MBD) has been recovered from marine (Vetriani et al., 1999) and lake sediments (Jurgens et al., 2000; Nusslein et al., 2001), and it is associated with RC-III and *Thermoplasma* groups (Kemnitz et al., 2005) found in anoxic soils. In this study we are proposing a new group of environmental phylotypes under the "Subaqueous Cluster" (SC) denomination. Members of this group were found at deep levels of CB, and were only associated with other sequences reported from submerged or deep environments (Nedelkova, 2005; Schrenk et al., 2003; Stein et al., 2002) and soil contaminated with material from subsurface

environments such as petroleum (Kasai et al., 2005). We speculate that SC members are specialized for survival in anaerobic, commonly submerged and highly reduced environments like deep layers of peat, lake sediments in deep sections, or deep wells. More studies are required to assess the distribution of SC and their role in these environments.

The results of 16S rRNA gene sequencing coupled to T-RFLP analyses allowed minimization of ambiguous peak identification. The T-RFLP parameters we developed are useful for studies on freshwater methanogenic populations, particularly in peatland ecosystems. Our results showed that the T-RFLP profiles of the upper peat layers at both sites were similar both in terms of community structure as well as physicochemical characteristics (Table 2-1 and Fig. 2-4). *Methanomicrobiales*-related phylotypes were resolved into three groups: E1, E1' and E2 by sequence clustering. E2 has also been called R10 cluster (Edwards et al., 1998) and Fen cluster (Galand et al., 2002) in other studies. E1' was only detected in clone libraries from deeper samples in CB (data not shown). *Methanospirillum hungatei* was the closest cultured organism to the E1 and E2 groups, but all three groups (E1, E2, and E1') represent novel methanogens whose physiology has yet to be elucidated.

Group E2 dominated T-RFLP profiles from the upper methanogenically active layers of both CB and MB, with smaller contributions from E1, similar to distributions in 16S rRNA gene clone libraries. Sequences in the E2 group have been found in an H₂/CO₂ enrichment (Sizova et al., 2003), and we have cultured a member of this group (6A in Fig. 2-3) on H₂/CO₂ (S. Bräuer et al., 2006). *Methanosaetaceae* and *Methanosarcinaceae* represented less than 10% of the total peak height in T-RFLP profiles, consistent with a modest contribution of methanogenesis from acetate to total methanogenesis. In studies of a neutral pH fen, these two groups made up > 50% of

the peak height (C. Sun, unpublished) indicating that the PCR primers and conditions were not biased against these groups.

The numerical dominance of hydrogenotrophic methanogens in the upper layers suggested by T-RFLP was confirmed with our real time PCR 16S rRNA gene target quantification, which estimated that the abundance of groups E2 and E1 together (although E1 contribution was small, particularly in CB samples) roughly accounted for ~ 55% of total archaeal gene targets in the upper layers, with their populations reaching a size on the order of 10^8 cells per gram of dry peat. These results clearly indicate the importance of group E2 for CH₄ production by CO₂ reduction in these *Sphagnum*-dominated sites. The E2 group is not unique to our study sites in northeast US, but instead has a widespread distribution in peatlands in the northern hemisphere. Phylotypes clustering within group E2 have been found in bogs from Germany (Horn et al., 2003), Finland (Galand et al., 2003), UK (Edwards et al., 1998), and Canada (J. Yavitt, unpublished), making up a significant fraction of clones in libraries or peak heights in T-RFLP analysis. An exception appears to be a pH 4.8 Siberian peat sample (Kotsyurbenko et al., 2004) in which E2 was only a small proportion of the methanogenic community. We speculate that this group is a significant contributor to methanogenesis in highly acidic peatlands. This hypothesis is in agreement with the significant presence of the “fen cluster” in Finnish peatlands (Galand et al., 2002; 2005; Juottonen et al., 2005) whose *mcrA* sequences are phylogenetically associated with the E2 culture (96% similarity, Bräuer et al., 2006). The population size and activity of group E2 at additional sites is required in order to confirm this findings.

In the vertical profiles, Group E2 decreased in proportion of methanogens in T-RFLP (Fig. 2-5) and in numbers (Fig. 2-6) at both sites in samples from 40 and 65 cm. These deep samples also showed low rates of endogenous and substrate stimulated

methanogenesis. In MB, E2 still represented about half of the total peak height, whereas it was barely detectable in the more neutral CB deep samples, consistent with its being associated with low-pH habitats. Group E2's preference for acidic conditions is supported by the observations of Bräuer et al (2004), where the pH optimum for peat incubations from MB was 4.8 in H₂/CO₂-amended treatments and that a shift to 5.5 or 6 strongly decreased CH₄ production.

Other significant changes in the community structure at depths below 40 cm were related to an increase in RC-II at both sites, and the presence of RC-I, MBD, SC and increase of E1 in CB. RC-II became an important fraction of the euryarchaeal community at both sites regardless of the pH differences, suggesting it has members that thrive in a broad range of pH values; this is supported by the fact that its sequences have been retrieved from acidic as well as neutral sites (Basiliko et al., 2003; Chin et al., 1999; Kemnitz et al., 2004). No isolated representative of RC, MBD or SC groups have been obtained up until now; although RC-I has been highly enriched with H₂/CO₂ from peat land and rice paddy soil samples with some initial physiological and genomic characterization (Erkel et al., 2005; Lueders et al., 2001; Sizova et al., 2003). It is interesting that the samples from CB lower levels, despite having low rates of methanogenesis, show considerably higher diversity than the upper levels, perhaps remnants of methanogenic populations present from a neutral pH fen.

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CHAPTER III
**ARCHAEAL COMMUNITY IN A MINEROTROPHIC FEN AND T-RFLP-
DIRECTED ISOLATION OF A NOVEL HYDROGENOTROPHIC
METHANOGEN ²**

3.1 ABSTRACT

Fen peatlands are widely distributed in northern latitudes and because of their rapid turnover of organic matter, are potentially larger sources of atmospheric CH₄ than bog peatlands per unit area. However, studies on the archaeal community composition in fens are scarce particularly in minerotrophic sites. Several 16S rRNA based primer sets were used to obtain a broad characterization of the archaeal community in minerotrophic fen in central New York State. A wide archaeal diversity was observed in the site: 11 euryarchaeal and 2 crenarchaeal groups most of which were uncultured. The E1 group, a novel cluster in the *Methanomicrobiales* order, and *Methanosaetaceae* were the co-dominant members in all libraries and T-RFLP analysis. Given its abundance and potential hydrogenotrophic CH₄ contribution, the E1 group was targeted for culture attempts with a low-ionic strength medium (PM1). Initial attempts yielded *Methanospirillum*-dominated cultures. However, by incorporating a T-RFLP analysis as a quick selection tool for treatments and replicates, we were able to select an enrichment dominated by E1. Further dilutions to 10⁻⁹ and tracking with T-RFLP yielded an isolated strain named E1-9c. E1-9c is a novel coccoid hydrogenotrophic, mesophilic, slightly acidophilic methanogen, and is

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highly sensitive to Na₂S concentrations (requires <0.12mM for growth). We propose E1-9c as the first representative of a novel genus in the *Methanomicrobiales* order.

3.2 INTRODUCTION

Peatlands are wetlands where the rate of accumulation of organic matter exceeds its rate of decomposition (Gore, 1983), producing organic peat soil. These ecosystems are estimated to globally store more than 30% of all terrestrial soil carbon (Gorham, 1991), and their anoxic and reducing conditions are suitable for CH₄ production by methanogenic *Archaea* (Wahlen, 1993). It is important to gain a better understanding of the processes and microorganisms involved in CH₄ production in peatlands because these ecosystems represent the largest natural sources of CH₄ to the atmosphere, levels of which have more than doubled in the past 200 years (Cicerone & Oremland, 1988). Moreover, peatlands are not homogeneous ecosystems but they include a wide range of sites that can substantially differ in vegetation, hydrology, and chemistry (Blodau, 2002, Turetsky & Ripley, 2005). The distinction between bogs and fens includes many of these differences.

Bogs (ombrotrophic or rain-fed sites) are dominated by *Sphagnum* mosses (Gorham & Jannsens, 1992), receive only atmospheric inputs of water, cations, and nutrients, and are nutrient-poor, low pH (≤ 4) ecosystems (Gore, 1983). In contrast, fens have a greater plant diversity, are commonly dominated by sedges and woody plants, receive the inputs of groundwater and runoff from surrounding uplands, and exhibit higher alkalinity and pH (5.5–7 in moderate minerotrophic fens; >7 in calcareous fens) (Gore, 1983, Vitt & Chee, 1990). Bogs are abundant in boreal regions and have been the focus of many studies on methanogenic processes, environmental controls and methanogenic *Archaea* (Avery et al., 1999, Blodau, 2002, Avery et al.,

2003, Juottonen et al., 2005, Bräuer et al., 2006a). However, fens have received far less attention despite their abundance and significant potential for CH₄ emissions.

Minerotrophic fens are widely distributed in subarctic regions (Roulet et al., 1992), western boreal forest of North America (Vitt et al., 2000), and other northern temperate zones (Wang et al., 1996, Bedford & Godwin, 2003). Additionally, minerotrophic fens have been found to have higher rates of CH₄ production (Yavitt et al., 1990, Bergman et al., 1998), higher overall CH₄ cycling with net fluxes ranging from consumption to high CH₄ emissions (Bridgham et al., 1998, Smemo & Yavitt, 2006, Keller & Bridgham, 2007), relatively rapid hydrology and short residence time compared to bogs (Smemo & Yavitt, 2006), and their highly-abundant vascular plants such as sedges and cattails facilitate the flow of CH₄ from deeper layers (Yavitt & Knapp, 1995, Popp et al., 1999). Thus, fens could have a greater global impact in CH₄ emissions than bogs in terms of CH₄ equivalents per unit area in the near future.

Only a few studies have characterized the archaeal community composition in fens from Northern peatlands. Studies have been done in oligotrophic or poor fens (Galand et al., 2002, Galand et al., 2003, Merila et al., 2006) as well as in mesotrophic ones (Galand et al., 2005, Juottonen et al., 2005), but minerotrophic fens have remained largely ignored. Nevertheless, the studies on poor fens and bogs (Galand et al., 2002, Basiliko et al., 2003, Kotsyurbenko et al., 2004, Juottonen et al., 2005) showed that *Archaea* are primarily associated to novel or uncultured groups in addition to some close relatives to known methanogens. Thus, we could expect that minerotrophic fens contain some of these or new uncultured groups.

Cultured representatives of these novel archaeal groups are desirable for understanding their ecology and role in peatlands. Although the cultivation of methanogens from peatlands have been proven difficult (Williams & Crawford, 1985), some recent attempts have succeeded enriching (Sizova et al., 2003, Bräuer et al.,

2006b) or isolating novel methanogens from bogs such as '*Candidatus Methanoregula boonei*' (Bräuer et al., 2006a) in the *Methanomicrobiales* as well as known methanogens from the *Methanobacteriales* (Kotsyurbenko et al., 2007).

The goal of this study was to characterize the archaeal community of a minerotrophic fen with particular emphasis on methanogenic *Archaea* and related groups. The community characterization showed that members of a uncultured group in the *Methanomicrobiales*, previously designated as E1 (Cadillo-Quiroz et al., 2006), were likely to be important in methanogenesis from H₂/CO₂. Members of E1 were targeted, and only after culturing efforts were coupled to molecular profiling using T-RFLP analysis, were we able to isolate a methanogen from the group. Phylotypes related to strain E1-9c have been found in minerotrophic fens, landfills, and anaerobic bioreactors, and the strain is proposed as a representative of a new genus in the order *Methanobacteriales*.

3.3 METHODS

Study site and sampling

Michigan Hollow (local name) is a 15-ha, minerotrophic fen near the village of Danby in central New York State, USA (42° 21' N, 76° 28' W), and was initially described by Bernard and MacDonald (1974). The peatland is located in the lower part of a small, forested watershed and receives surface and subsurface water flow from the surrounding forested uplands (Smemo & Yavitt, 2006). The vegetation at the site is currently dominated by *Carex lacustris* (lake sedge), *Typha latifolia* (common cattail), and *Lythrum salicaria* (purple loosestrife). Triplicate peat samples were collected anaerobically at a 20 cm depth in April 2005 using the same procedures previously described (Bräuer et al., 2004). Samples were processed inside of an anaerobic glove box (Coy, USA) no later than 2 hours after sampling.

16S rRNA gene amplification, cloning and phylogenetic analysis

DNA from peat samples and enrichment cultures were extracted in duplicate with the Power SoilTM DNA or Ultraclean Microbial DNA extraction kits respectively (MoBio, USA), using the manufacturer's protocol. PCR amplifications were performed as previously described (Cadillo-Quiroz et al., 2006). Amplifications were done with three archaeal primer combinations and a eubacterial one with their corresponding annealing temperatures: 1Af-1100r (Hales et al., 1996) at 50°C, 1Af-1492r at 50°C, 1Af-ArchLSU47 (Bräuer et al., 2006a) at 53°C, and 27f-1492r at 50°C. PCR products were examined by electrophoresis on 1% agarose gels.

Two clone libraries for each of the three archaeal primer combinations were constructed as previously described (Basiliko et al., 2003, Cadillo-Quiroz et al., 2006). Sequences were compared against the GenBank database to ensure that newly-reported relatives were included in the analysis. The alignment of sequences was done with the ARB software (Ludwig et al., 2004) and exported from it using a nucleotide base frequency filter including positions with more than 50% invariance (1420 valid columns). Trees were constructed by Bayesian analyses using MrBayes (version 3.0 (Ronquist & Huelsenbeck, 2003)) and four-chain Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analysis. Chains commonly converged after 1,000,000 generations. Bayesian consensus tree was built with a burnout of 300 and nodes with ≥ 0.80 posterior probability were considered significant. Trees were imported back to ARB and smaller sequences were added using the parsimony tool without altering global tree topology. Tree topology was confirmed using maximum likelihood and neighbor joining methods (implemented in ARB) with Olsen evolutionary distance correction. The presented tree represents the most frequently observed topology across various phylogenetic analyses.

Putative chimeras were identified, and excluded from phylogenetic analysis, using the Bellerophon server (Huber et al., 2004). Rarefaction analysis of sequences was done using DOTUR (Schloss & Handelsman, 2005) with the furthest neighbor assignment method. 16S rRNA, Intergenic Transcribed Sequence (ITS), and 23S rRNA gene sequences were deposited in the GenBank nucleotide sequence database under the accession numbers EU155896 to EU155999 for environmental clones, and EU156000 for strain E1-9c.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP analysis was performed as previously described (Cadillo-Quiroz et al., 2006) with the 1Af-1100r primers and the reverse primer fluorescently labeled on its 5' end with Carboxyfluorescein (5'-/6-FAM). Fragments were resolved using an Applied BioSystems Gene Scan 500-Liz marker with a 3730xl DNA Analyser (Bio Resources Center, Cornell University). Terminal restriction fragment (T-RF) sequence length, peak height and area were determined using the GeneScan Analysis Software (Version 3.1. Applied Biosystems, USA). To exclude potential pseudo T-RFs, traces were standardize to a total of 100 units and peaks below the arbitrary value of 0.5 were eliminated from the analysis.

CH₄ production by peat slurries

Peat slurry incubations with or without substrate additions were performed as previously described (Bräuer et al., 2004). Susbtrate additions were done with anaerobic stock solutions to the following final concentrations: sodium acetate (1 mM), rifampicin (10 mg L⁻¹), which was added to impede growth of acetogens in H₂/CO₂ enrichments (Bräuer et al., 2004) and to corresponding controls without H₂/CO₂. Sterile O₂-scrubbed H₂/CO₂ (80%/20%, 70.7 kPa, Mixed Gas Industries,

USA) was added to appropriate tubes. Presented data represent the averages of triplicate samples.

Chemical analyses

CH₄ contents in headspace gas samples were analysed using a Perkin Elmer 3920B gas chromatography column with a flame ionization detector (Phoenix Equipment, USA) as previously described (Bräuer et al., 2004). Peat slurries were vortexed prior to headspace analysis.

Growth of methanogenic culture

Culturing efforts were done using the low-ionic-strength medium PM1 as previously described (Bräuer et al., 2006a, Bräuer et al., 2006b) with the following anaerobic additions to their final concentrations: 1.0 mM titanium (III) nitrilotriacetate (Moench & Zeikus, 1983), 10 mM MES (2-(N-morpholino) ethane sulfonic acid ($pK_a = 6.2$ at 28 °C, 1 M stock solution adjusted to pH 7), 0.5 mM coenzyme-M (2-mercapthoethanesulfonic acid), 0.5 mM sodium acetate, 100 µM FAM (fatty acid mixture: isobutyric, valeric, isovaleric and DL-2- methyl butyric acid), 0.1 g L⁻¹ yeast extract, 1% v/v vitamin solution (Balch et al., 1979), 1 µM H₂S (added as sterile gas), and 10 mgL⁻¹ rifampicin. The final liquid volumes in the tubes were ca. 5 ml, and 70.7 kPa H₂/CO₂ was added to the headspaces. The cultures were incubated in a shaker (200 r.p.m) at 28°C. After obtaining a culture from a 10⁻⁹ dilution, tests were done to determine which additions were strictly required. Yeast extract, FAM, and rifampicin were not required for E1-9c growth and consequently were excluded in further culture transfers and tests.

For experiments on the effect of pH, the pH was adjusted by addition of 30 mM MES adjusted to various pH values. Low pH evaluations were also attempted by adding 5 mM of either Homopipes or citric acid instead of MES. The pH of the cultures was assessed at the end of incubations. For the Na₂S sensitivity test, Na₂S.9H₂O filter-sterilized anaerobic solutions were prepared with different concentrations so the same volume of reagent (0.05 mL to 5 mL cultures) was added to replicate cultures. For experiments on the effect of temperature, cultures were incubated under static conditions. All the tests were performed in triplicate and repeated at least once. Growth rates were calculated from CH₄ production from the exponential parts of the CH₄ accumulation curve.

Bright-field microscopy, Fluorescence in situ hybridization (FISH), and electron microscopy

A Nikon Eclipse E600 microscope equipped with a Hamamatsu CCD digital camera was used for light and fluorescence microscopy. For examination of cells by FISH, cells were fixed in 4% paraformaldehyde for 15-24 hours, filtered onto black 0.2 µm polycarbonate membrane filters, and stored with desiccant at -20°C. FISH was performed as described by Morris et al. (Morris et al., 2002). Hybridization buffer contained no formamide and 2 µg mL⁻¹ DAPI (4', 6-diamidino-2-phenylindole) and a indocarbocyanine dye-labeled (Cy-3-labeled) probe at a final concentration of 2 ng µL⁻¹ (ARCH 915 or EUB 338 (Amann et al., 1990)).

Negative staining transmission electron microscopy was performed as follows: 5µL of cells resuspended in deionized water were mixed with a drop of bacitracin (100 µg mL⁻¹) and allowed to settle onto Formvar-coated copper grids (100 mesh) for 5 minutes. Staining with 2% uranyl acetate (pH 6.5) was done for a few seconds.

Micrographs were acquired with a Phillips EM-201 electron microscope and a Gotan model 780 camera at 100 KV.

3.4 RESULTS

Archaeal diversity assessed by 16S rRNA gene analysis.

The 16S rRNA gene clone libraries revealed a diverse archaeal community on the shallow layer of Michigan Hollow fen (Fig. 3-1 and 3-2). Some euryarchaeal phylotypes were associated with known methanogen clades such as *Methanosaetaceae* (MT), *Methanosarcinaceae* (MS), *Methanobacteriaceae* (MB), and *Methanospirillaceae* (MP). Other phylotypes were related to uncultured groups such as “group E1”, “Subaqueous Cluster” (SC), “group E1’ ” (E1’), Marine Benthic group D (MBD), Rice Cluster II (RC-II), or recently-cultured groups such as “E2/*Methanoregula*” (E2) (Bräuer et al., 2006a) and Rice Cluster I (RC-I) (Sakai et al., 2007). Crenarchaeal phylotypes that belonged to the uncultured groups “Rice Cluster IV” (RC-IV) and “VI” (RC-VI) were also recovered (Fig. 3-1).

The clones associated with groups E1 and MT were numerically dominant in all libraries regardless of the primer combination, with an average abundance of 35% and 39% respectively. The other archaeal groups always represented smaller fractions in the libraries (Fig. 3-2). The dominant E1 and MT groups were similarly well covered by the different primer combinations. However, differences in coverage were observed in the detection of minority groups. The 1AF-ArchLSU47 combination did not recover sequences from the RC-I, RC-II, MBD, MP and MB groups, although its libraries contained fewer clones than the other two primer sets (Fig. 3-2).



Figure 3-1. Phylogenetic analysis of archaeal 16S rRNA gene clones from Michigan Hollow fen. The abbreviations of the recovered groups are as follows: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT); subaqueous cluster (SC); rice cluster-I (RC-I); rice cluster-II (RC-II); group E1 (E1), group E1' (E1'), group E2 (E2) (classification based on Cadillo-Quiroz et al., 2006 and Basiliko et al., 2003), *Methanospirillaceae* (MP); *Methanobacteriaceae* (MB); marine benthic group D (MBD) (based on Vetriani et al., 1999); rice cluster-IV (RC-IV) and rice cluster-VI (RC-VI).

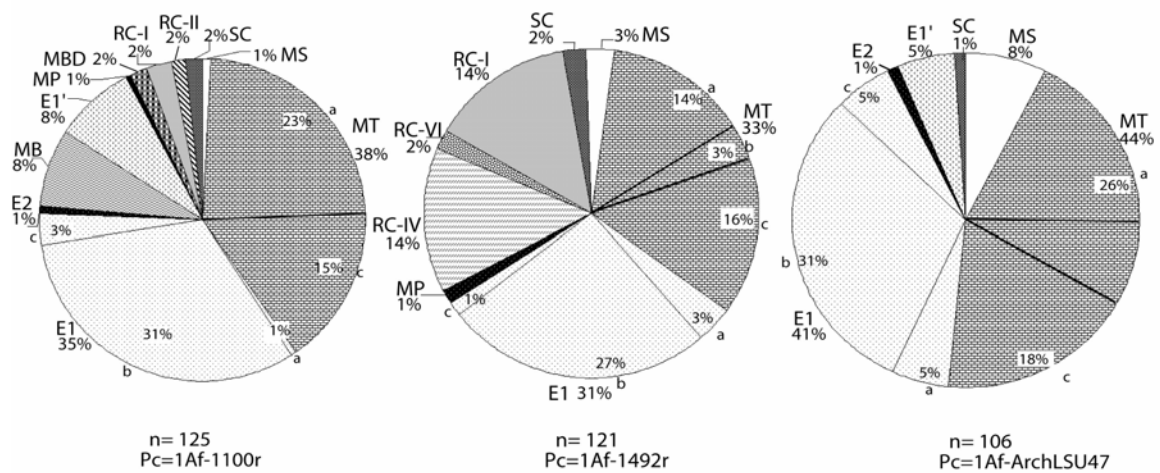


Figure 3-2. Clone distribution from libraries constructed with different primer combinations. The total number of clones (n) and primer combination (pc) are indicated for each primer set. The abbreviations of the recovered groups are as follows: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT); subaqueous cluster (SC); rice cluster-I (RC-I); rice cluster-II (RC-II); group E1 (E1), group E1' (E1'), group E2 (E2), *Methanospirillaceae* (MP); *Methanobacteriaceae* (MB); marine benthic group D (MBD); rice cluster-IV (RC-IV) and rice cluster-VI (RC-VI).

The 1AF-1492r primer set did not recover the groups RC-II, MB, MBD and E1'. However, this set recovered several crenarchaeal sequences (RC-IV and VI) that made up 16% of these libraries (Fig. 3-2). The rarefaction analysis of operational taxonomic units (OTUs) at 97.5% sequence identity (Fig. 3-3) indicated that 1Af-ArchLSU47r has a narrow scope (close to sampling saturation with only 8 OTUs), and that 1AF-1492r and 1Af-1100r primers hit similar numbers of groups (18 and 17) with some differences in the amount of sampling required to approach saturation on their coverage. The collective analysis of the libraries showed that although each primer combination could be close to reach its saturation, the total Archaeal diversity is significantly higher and far from each of their hit values as evidenced when comparing them to the total of 31 OTUs (Fig. 3-3).

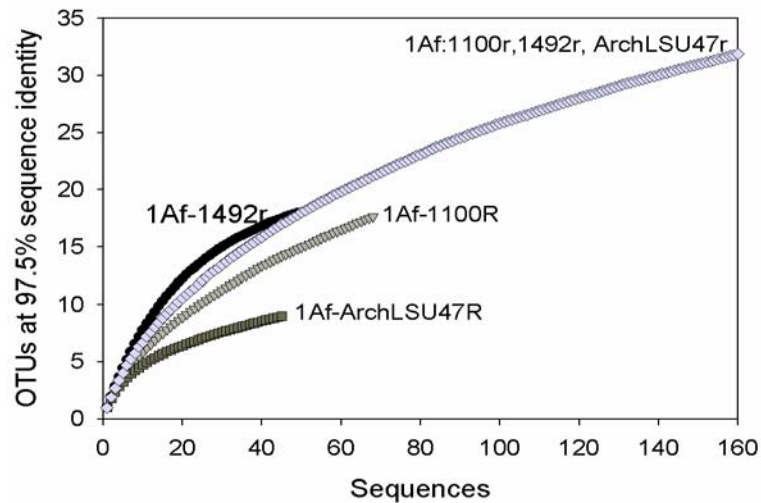


Figure 3-3. Rarefaction analysis of Michigan Hollow fen clone libraries. Analysis is shown for libraries from each corresponding primer mix and for all libraries collectively (indicated). 97.5% sequence identity was chosen as the arbitrary value for defining the operational taxonomic unit (OTU) for this comparison

Additionally, the sequences from group E1 and MT showed phylogenetically robust clusters (underlined bootstrap values in Fig. 3-1), containing 3 clusters each when using a 97.5% similarity cut off value. Some phylotypes in cluster E1-c (Fig. 3-1) did not satisfy our similarity criterion; but because they were represented only by single sequences, they were provisionally classified as part of this single cluster. Similar patterns of clusters were observed when the intergenic transcribed sequence (ITS) region amplified by the 1AF-ArchLSU47 combination was included in the sequence analysis (not shown). None of the E1 clusters contained a sequence from a cultured organism, whereas only 'Cluster a' in MT contained sequences from cultured *Methanosaeta* strains (Fig. 3-1).

CH₄ production by peat soil incubated with substrates and T-RFLP analysis

CH₄ production (Fig. 3-4) was similar among all treatments in the initial 10 days of incubation, but after 14 days the acetate and H₂-CO₂/rifampicin (rif)-amended samples produced more CH₄. The euryarchaeal community structure of peat samples

was analyzed by T-RFLP analysis using the 1Af-1100r primers (Fig. 3-5). Peak identity was predicted by *in silico* digestion of our clone libraries as described by Cadillo-Quiroz et al (2006). *Methanospirillum* (MP) was the only member from the group E2' (Cadillo-Quiroz et al., 2006) with sequences recovered from MH, and thus MP instead of E2' was used for the identity of the peak. T-RFLP profiles of peat samples before incubation (Fig. 3-5.A and 3-5.B.1) showed E1 and MT as the dominant peaks along with smaller peaks predicted to represent groups such as MS, MB, E2-MP, RC-I/SC and RC-II, in agreement with the clone libraries.

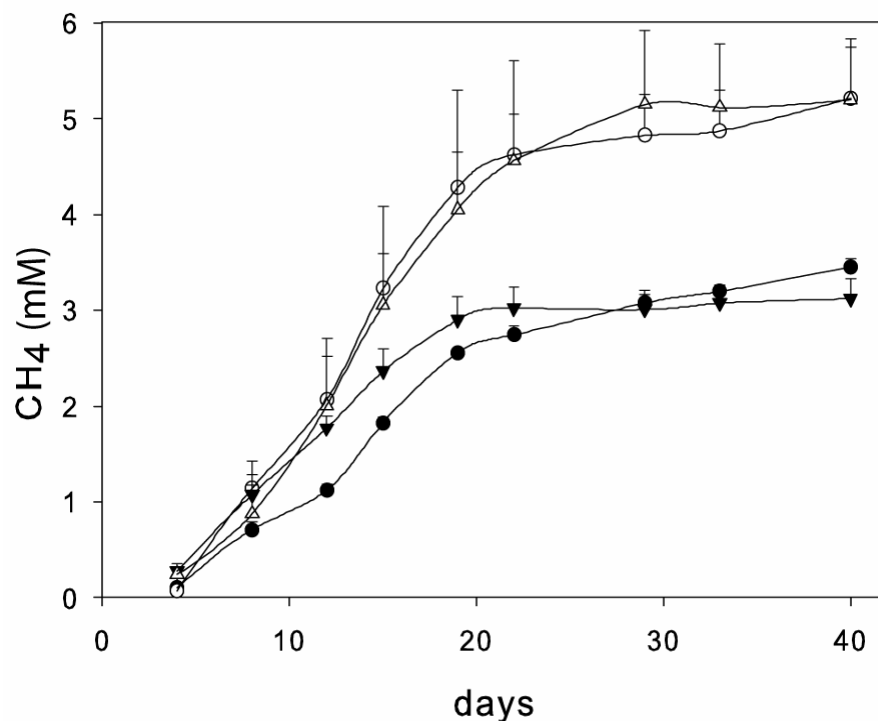


Figure 3-4. CH₄ production by peat slurries from Michigan Hollow fen. Samples were non-amended static (white circles), or static amended with: 1mM acetate (black circles), shaken and amended with rifampicin (black triangles) or shaken and amended with H₂/CO₂ and rifampicin (white triangles). Points represent the mean± SD, n=3.

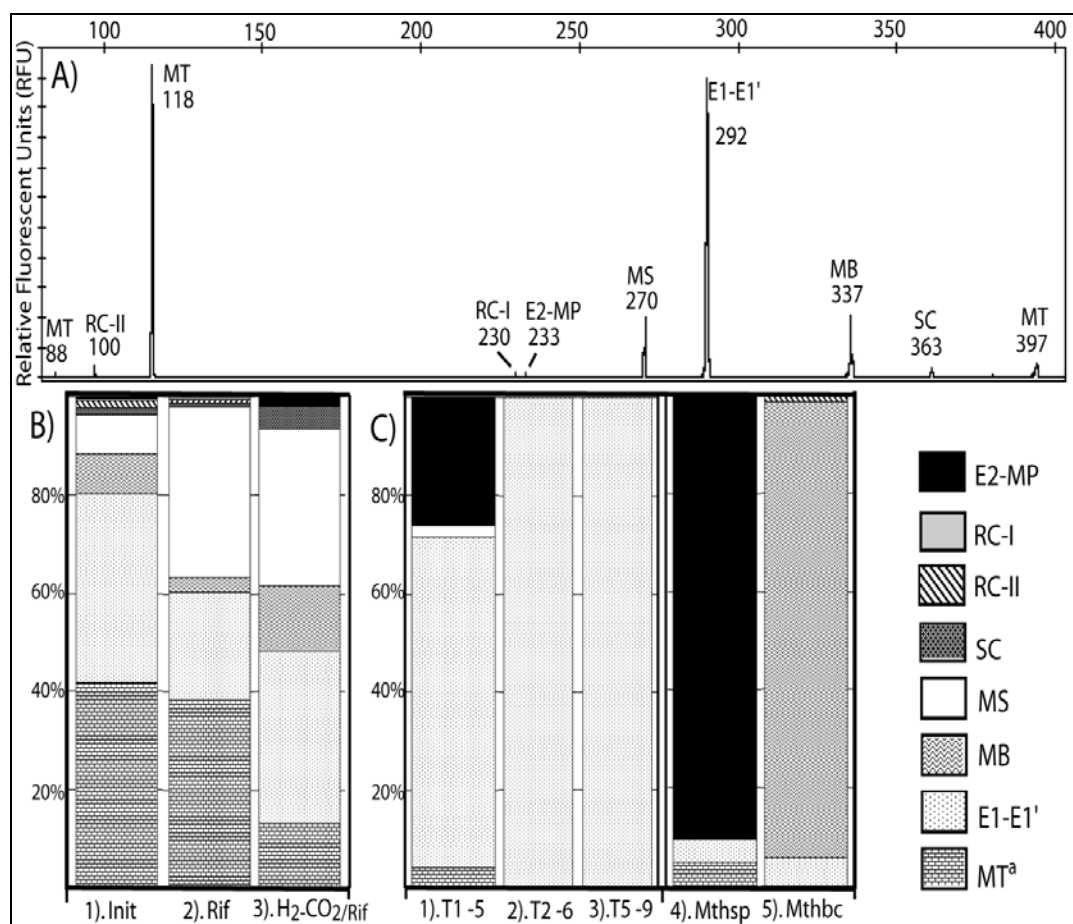


Figure 3-5. T-RFLP analysis of peat samples and enrichments using the 1Af-1100r primer set and HhaI-Sau96 restriction enzymes. T-RFLP profiles (A) were standardized to a total of 100 relative fluorescent units (RFU), and the peaks matched with its correspondent group are presented in a single column format (B and C). Initial (Init) or no-incubated samples are grouped with incubated slurries (A and B) amended with rifampicin-only (rif) or H₂-CO₂/rifampicin (H₂-CO₂/rif). Enrichment cultures (C) profiling was done in the highest reached dilution of each E1-targeted culture transfer: transfer 1 dilution 10⁻⁵ (T1-5), transfer 2 dilution 10⁻⁶ (T2-6), transfer 5 dilution 10⁻⁹ (T5-9); other enrichments attempts that yielded *Methanospirillum* (Mthsp) and *Methanobacterium* (Mthbc) related organisms were also included. The groups abbreviations are as follow: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT); subaqueous cluster (SC); rice cluster-I (RC-I); rice cluster-II (RC-II); group E1 (E1), group E1' (E1'), group E2 (E2), *Methanospirillaceae* (MP); *Methanobacteriaceae* (MB).

End point analysis of peat samples incubated shaking and amended with rifampicin but without H₂-CO₂ (Fig. 3-5.B.2), showed an increase in the proportion of the peak corresponding to MS with a corresponding reduction in the E1 and MB. A

smaller increase in the MS peak was found in cultures incubated statically without rifampicin (data not shown). Meanwhile, in the H₂-CO₂/rifampicin-amended peat, E1 remained as the dominant peak (Fig. 3-5.B.3), while MB and MS also increased their proportions, and MT was significantly decreased.

T-RFLP-assisted isolation of a novel methanogen

Several attempts to isolate a member of the E1 group were made using the low-ionic-strength medium PM1 (Bräuer et al., 2006a, Bräuer et al., 2006b) adjusted to higher pH (ca. 5.5-6) to reflect fen conditions. The initial attempts using fresh or incubated peat and dilution to extinction transfers, in PM1 plus H₂-CO₂, yielded *Methanospirillum*-dominated or *Methanospirillum*-only cultures (data not shown).

In order to target the isolation of a member of the E1, a euryarchaeal-specific T-RFLP profiling was used to assess culturing conditions or replicated samples where E1 increased its proportion. The H₂-CO₂/rifampicin incubated peat (Fig. 3-5.B.3) was used to inoculate replicated sets of PM1 plus H₂-CO₂ with different buffers (Homopipes or MES), different pH values (4.5 or 5.6) and different incubation temperatures (28 or 34°C). A set of tubes in the treatment with MES, pH 5.6, and 28°C produced CH₄ in dilutions as high as 10⁻⁵. T-RFLP profiling showed that in these 10⁻⁵ tubes, E1 increased its fraction to around 70% while MS and MT were less than 9%. *Methanospirillum* (MP) was also significantly present making up around 21% of the total profile (Fig. 3-5.C.1). The 10⁻⁵ enrichment was transferred to a new round of dilutions and CH₄ production was observed in 10⁻⁶ dilutions. T-RFLP analysis of a sample from this dilution indicated that E1 was the only euryarchaeal group detectable by our 1Af-1100r primers (Fig. 3-5.C.2). We performed four more dilution rounds,

obtaining growth (turbidity) and CH₄ production in 10⁻⁹ dilutions. T-RFLP analysis indicated that E1 was the only group present in the culture (Fig. 3-5.C.3).

A set of PM1 tubes, using the same conditions that successfully enriched for the E1 were inoculated with unincubated peat and produced CH₄ up to the 10⁻³ dilution, but the T-RFLP profile showed that *Methanospirillum* (MP) was the primary enriched group (Fig. 3-5.C.4). Microscopy observations of this enrichment confirmed the dominance of *Methanospirillum*-like cells (not shown). Other treatments, such as Homopipes, pH 4.5, and 34°C incubation, produced CH₄ in dilutions only as high as 10⁻² and T-RFLP of this dilution indicated a strong enrichment of the MB group (Fig. 3-5.C.5). These two treatments were not further pursued since E1 was not preferentially enriched.

Purity and initial characterization of isolate E1-9c

The culture that passed through a 10⁻⁹ dilution was designated strain E1-9c, contained cells with a single morphology (Fig. 3-6A), and several tests were performed to assess its purity. Organic substrates such as yeast extract (0.2 g L⁻¹), glucose, pyruvate and lactate (20 mM) were added to the culture in the absence of rifampicin to test for heterotrophic contaminants, and no growth or methanogenesis was observed. Attempts at PCR amplification with universal bacterial 16S rRNA gene primers 27F-1492r were also unsuccessful. Fluorescent *in situ* hybridization (FISH) with the universal archaeal probe ARCH915 showed hybridization with all the cells in the culture (Fig. 3-6C), and no hybridization with the bacterial probe EUB 318 (not shown).

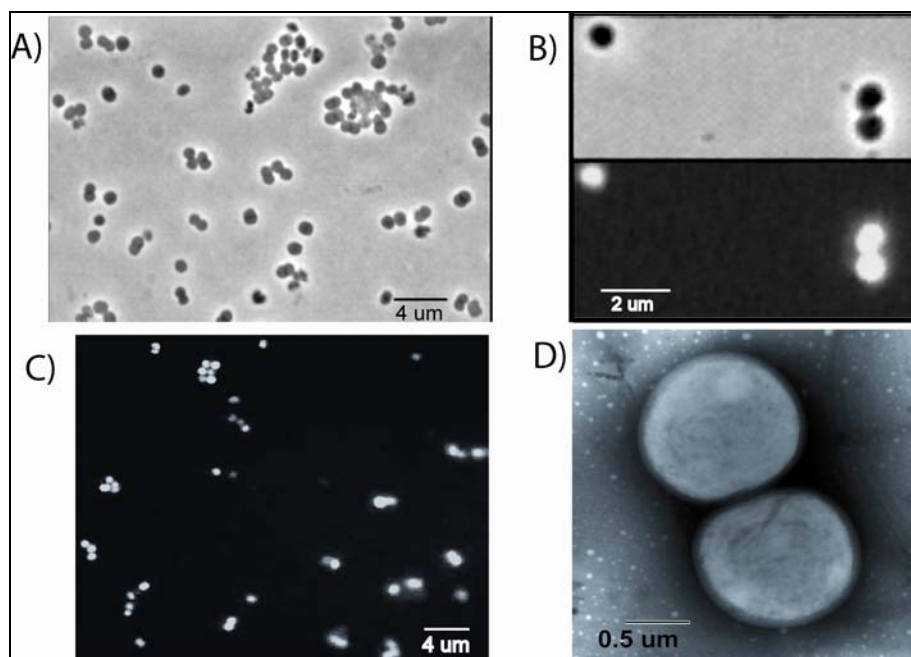


Figure 3-6. Microscopic examination of E1-9c cells. Phase-contrast microscopy (A); phase-contrast and fluorescence microscopy showing the autofluorescence of cells illuminated with light near 420 nm (B); FISH using the 915-Arch probe (C); negative stain electron microscopy (D).

Additionally, we constructed a clone library with the primers 1AF-ArchLSU47. All 70 clones had the same restriction pattern and the three sequenced clones had only three base differences out of a 1784 bases, well within the margin of error for PCR amplifications (von Witzingerode et al, 1997). From these observations the purity of the E1-9c culture was established. Complementary, the phylogenetic analysis of the full 16S rRNA gene sequence positioned E1-9c in the order *Methanomicrobiales* forming a unique cluster closely related to environmental sequences of the group E1-c (Fig. 3-1). E1-9c closest described cultured relatives are '*Candidatus* Methanoregula boonei' and *Methanospirillum hungatei* (93% and 92% identity respectively).

Strain E1-9c cells had a coccoid morphology with a diameter of 0.5-0.8 µm and were often in pairs (Fig. 3-6). Cells were non-motile and showed strong blue

fluorescence when illuminated with light near 420 nm, indicative of abundant coenzyme Factor 420 (F_{420}) in the cells (Figure 3-6.B). E1-9c used H_2 - CO_2 as methanogenic substrate with a doubling time of ca. 2 days at pH 5.6 and 28°C (standard growth conditions). In addition to mineral nutrients, E1-9c also required vitamins solution (Balch et al., 1979), coenzyme M (0.5 mM), and acetate (0.4 mM; as carbon source) in the growth medium.

CH_4 production by E1-9c was observed from 7°C to 37°C with an optimum near 30°C (Fig 3-7.A). CH_4 production above half its maximum rate occurred between 24°C and 35°C, indicating the mesophilic nature of the isolate. E1-9c grew from pH 4.8 to 6.5, with an optimum of 5.3-5.5 (Fig. 3-7.B). pH values lower than 4.8 could not be obtained using MES buffer. We attempted to use Homopipes and citric acid as low-end pH buffers, but no growth was observed even at similar pH values where growth was observed using MES, which suggests some detrimental effects of these buffers on the growth of strain E1-9c.

The effects of H_2S or $Na_2S \cdot 9H_2O$, a common reducing agent and sulfur source in anaerobic growth media, on the growth of strain E1-9c was examined (Fig. 3-7.C). Very low concentrations of Na_2S (0.01 to 0.08 mM) improved the growth of E1-9c compared to our standard H_2S gas addition (~0.001 mM). The optimum Na_2S addition was around 0.04 mM, and additions above 0.12 mM completely inhibited the growth of strain E1-9c.

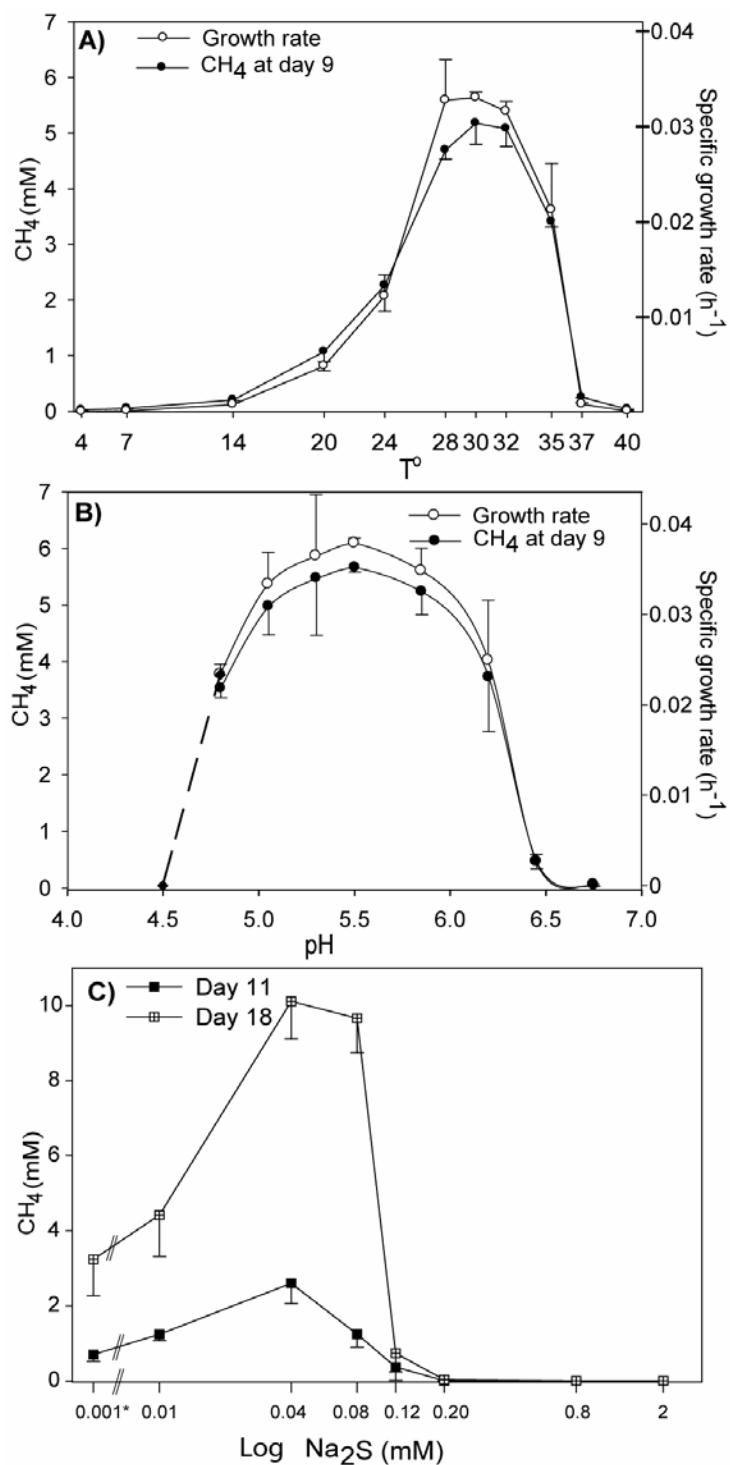


Figure 3-7. Physiological characterization of E1-9c. Effect of temperature on growth and methanogenesis (A); effect of pH on growth and methanogenesis (B); effect of sulfide (Na₂S) on methanogenesis (C). (*) represent a set of tubes with H₂S gas addition instead of Na₂S (H₂S gas was initially used for the isolation of E1-9c). Points represent the mean \pm SD, n=3.

3.5 DISCUSSION

Michigan Hollow (MH), is a minerotrophic fen where multi-year observations of CH₄ fluxes have shown significant CH₄ emissions to the atmosphere particularly in rainy years (Smemo & Yavitt, 2006). Minerotrophic fens, such as MH, are abundant in the boreal forest biome particularly in North America (Bedford & Godwin, 2003, Turetsky & Ripley, 2005); nevertheless, the methanogenic communities inhabiting these fens have scarcely been characterized. To better characterize the euryarchaeal community from the MH fen site, multiple clone libraries were constructed using different primer combinations targeting the 16S rRNA gene (Fig. 3-1 and 3-2). Individually each primer combination achieved near sampling saturation but with only a 58 to 26% coverage of all OTU's at 97.5% sequence identity (Fig. 3-3), suggesting that a multiple primer approach is required for a thorough community characterization. The libraries showed a diverse community where the majority of phylotypes are associated with uncultured groups (Fig. 3-1 and 3-2). The recovered phylotypes were associated to a total of eleven euryarchaeal and two crenarchaeal groups as follows: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), Subaqueous Cluster (SC), Rice Cluster I (RC-I), Rice Cluster II (RC-II), group "E2/Methanoregula" (E2), group "E1" (E1), *Methanospirillaceae* (MP), group "E1'" (E1'), *Methanobacteriaceae* (MB), Marine Benthic group D (MBD), Rice Cluster IV (RC-IV) and Rice Cluster VI (RC-VI) (Fig. 3-1 and 3-2). Although this report had a more extensive number of screened clones (352 total in Fig. 3-2) and used different primers than other studies; the Euryarchaeal composition in the fen was more diverse than what has been observed in some other bogs, oligotrophic fens, or mesotrophic fens (Galand et al., 2003, Basiliko et al., 2003, Kotsyurbenko et al., 2004, Rooney-Varga et al., 2007). This observation has mixed support from the comparison of rarefaction analysis of clone libraries from

other studies (Galand et al., 2003, Basiliko et al., 2003, Kotsyurbenko et al., 2004, Cadillo-Quiroz et al., 2006). Clone libraries from two bogs and one oligotrophic fen showed lower diversity than MH fen while results from a Siberian bog suggest similar diversity (Fig. 3-8). More clone libraries using similar sampling efforts (number of clones) and primers would facilitate the comparisons of the 16S rRNA gene diversity from different sites.

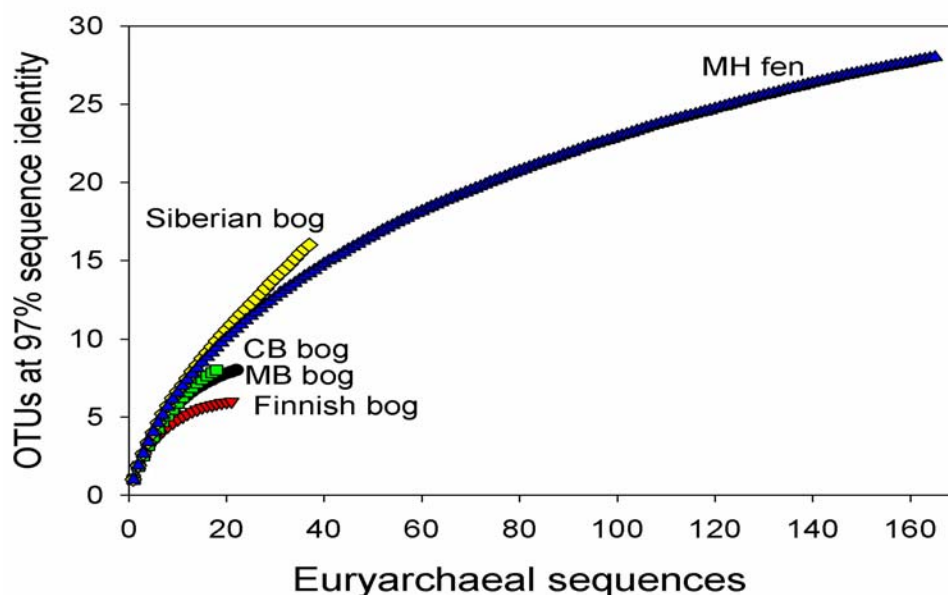


Figure 3-8. Rarefaction analysis of Michigan Hollow fen and other peatland clone libraries. Sequences from Finnish bog (Galand et. al 2003), McLean Bog NY (MB) (Basiliko, et al., 2003), Chicago Bog NY (CB) (Cadillo-Quiroz, et al., 2006) and Siberian Bog (Kotsyurbenko, et al., 2004) were obtained from GenBank database and aligned with ARB software. Distance matrixes were calculated with ARB using a nucleotide base frequency filter including positions with more than 50% invariance. Rarefaction analysis was done with DOTUR with the furthest neighbor assignment algorithm. 97% sequence identity was chosen as the arbitrary value for defining the operational taxonomic unit (OTU) for the comparison.

In terms of primers, the 1Af-1100r combination (Hales et al., 1996) amplified all euryarchaeal groups detected by the different mixes, making this set optimal for euryarchaeal coverage in MH (Fig. 3-2). The 1Af-1492r and 1Af- ArchLSU47 primers missed sequences of ca. 5 minor groups each (Fig. 3-2), and the 1Af-1492r mix was the only one that amplified crenarchaeal sequences in addition to euryarchaeal ones

(Fig. 3-3). The 1AF- ArchLSU47 primer mix, allowed the recovery of full 16S and ITS rRNA gene sequences of many euryarchaeal groups in MH, although RC-I sequences were not recovered. rRNA gene sequences from the RC-I genome (Erkel et al., 2006) had perfect matches with the 1Af- ArchLSU47 primers but the size of its ITS regions, 582 bases in two and 344 bases in one (Erkel et al., 2006), would lead to fragments larger than 2 Kb that are less efficient to amplify or clone with standard techniques. Fragment size could represent a limiting factor in the coverage of 1Af- ArchLSU47 primers. Nevertheless, the retrieval of a large fragment of the rRNA operon (~1.75 KB average size) allowed the first examination of the variability (size and sequence) of the ITS regions of uncultured *Euryarchaea*, including the presence or absence of tRNA (Table 3-1), in addition to providing full 16S rRNA sequences for phylogenetic analysis and primer or probe design to study novel groups such as Subaqueous Cluster, E1 group or E1' group. Only a previous study has examined the ITS variability of uncultured *Archaea*, and it examined marine crenarchaea (Garcia-Martinez & Rodriguez-Valera, 2000).

Multiple phylotypes associated with several uncultured euryarchaeal groups were recovered. Groups such as RC-I, RC-II, RC-IV, RC-VI (Grobkopf et al., 1998) and MBD (Vetriani et al., 1999) have also been found in bogs (Sizova et al., 2003, Basiliko et al., 2003, Kotsyurbenko et al., 2004, Cadillo-Quiroz et al., 2006), poor fens (Galand et al., 2002, Juottonen et al., 2005) and lake sediments (Jurgens et al., 2000, Glissman et al., 2004).

Table 3-1. Intergenic Transcribed Sequence (ITS) size and tRNA presence in Euryarchaeal phylotypes and associated cultured methanogens. Ala: Alanine, Gln: Glycine and Ser: Serine.

	tRNA	ITS size	Group
<i>Methanosarcina acetivorans</i> (AE010299)	no, no, no	282, 329, 141	<i>Methanosarcinaceae</i>
<i>Methanosarcina barkeri</i> st Fusaro	no, no, Ala	218, 218, 310	
MHLSU47_111G	no	187	
MHLSU47_B1A	no	268	
MHLSU47_13B	no	212	
MHLSU47_15C	no	260	
MHLSU47_4B	Ala	360	<i>Methanosaetaceae-a</i>
MHLSU47_11C	Ala	379	
MHLSU47_B12D	Ala	378	
MHLSU47_B3E	Ala	343	
MHLSU47_1E	Ala	339	
MHLSU47_6E	Ala	314	
MHLSU47_B8F	Ala	321	
MHLSU47_7F	Ala	328	
MHLSU47_7G	Ala	323	
MHLSU47_21D	Ala	372	
MHLSU47_11F	Ala	371	
MHLSU47_2B	Ala	340	<i>Methanosaetaceae-c</i>
MHLSU47_22D	Ala	333	
MHLSU47_B12A	Ala	328	
MHLSU47_B12C	Ala	330	
MHLSU47_B1H	Ala	329	
MHLSU47_B1E	Ala	330	
MHLSU47_23F	Ala	331	<i>Methanosaeta thermoacetophila</i>
<i>Methanosaeta thermoacetophila</i>	Ala, Ala	251, 251	
RC-I (NC_009464)	Ala, Ala, no	582, 582, 344	Rice cluster
MHLSU47_B11G	Ala	334	Subaqueous cluster
MHLSU47_17C	no	237	Group E2
MHLSU47_10H	no	231	Group E1-a
MHLSU47_12F	no	239	
MHLSU47_11F	no	232	
MHLSU47_7C	no	235	
MHLSU47_29A	no	241	Group E1-b
MHLSU47_1G	no	243	
MHLSU47_1F	no	235	
MHLSU47_3E	no	241	
MHLSU47_B4B	no	238	
MHLSU47_9A	no	240	
MHLSU47_10C	no	239	
MHLSU47_11B	no	240	
MHLSU47_B3H	no	239	
MHLSU47_1B	no	240	
MHLSU47_15E	no	240	
MHLSU47_B6B	no	240	
MHLSU47_B4A	no	243	
MHLSU47_4H	no	234	Group E1-c
Strain E1-9c	no	252	
MHLSU47_B11B	Gln	244	
MHLSU47_B8A	Ser	242	
<i>Methanospirillum hungatei</i> (CP000254)	Ala, Ala, Ala, Ala	300 (x4)	<i>Methanospirillaceae</i>
MHLSU47_1A	Ala	240	Group E1'
MHLSU47_14G	Ala	240	
MHLSU47_29F	Ala	239	

The abundance of these groups varies in peatlands from modest to significant fractions in clone libraries, but information about their role or physiology is limited by the lack of cultures. A member of the RC-I group was recently isolated (Sakai et al., 2007), confirming the group as H₂-CO₂ utilizing methanogen, as observed in previous enrichments (Sizova et al., 2003). The RC-I group is particularly abundant in rice paddies where it can make up 20 to 50% of total methanogens (Kruger et al., 2005); however in the MH fen, this group represented only 2 to 14% of the clones libraries. RC-I have been suggested to be well adapted to low H₂ habitats (Sakai et al., 2007) and relatively oxic conditions (Erkel et al., 2006) such as the rice rhizosphere where RC-I phylotypes have been commonly found (Grobkopf et al., 1998). RC-I could play a role in the rhizosphere of sedges in minerotrophic fens; however, this would require further investigation, since our study focused on bulk peat.

Groups E1' and SC have recently been identified in peatlands (Cadillo-Quiroz et al., 2006) and some associated phylotypes have been observed in other ecosystems. Group E1', associated to the order *Methanomicrobiales*, has until now only been found in forested and acidic peatlands from NY (Basiliko et al., 2003, Cadillo-Quiroz et al., 2006). At this site E1' made up only 5 to 8% of the clones, suggesting a small population size, which could make its detection difficult in low coverage libraries. In contrast, phylotypes related to the SC group have been found in lake sediments, contaminated soils and nitrate-rich canal sediments (Stein et al., 2002, Kasai et al., 2005, Raghoebarsing et al., 2006). Interestingly, a sequence affiliated to SC (D-arch in Fig. 3-1) was shown as the unique archaeal partner in an enrichment from nitrate rich-sediments, performing anaerobic oxidation of methane (AOM) coupled to denitrification (Raghoebarsing et al., 2006). Whether members of the SC group play a role in AOM in freshwater sites like Michigan Hollow is unknown.

CH₄ production among H₂-CO₂ or acetate-amended and non-amended samples (stimulated and endogenous methanogenesis respectively) was similar in the initial days of incubation (Fig. 3-4), suggesting that both aceticlastic and hydrogenotrophic methanogens were active and not substrate-limited in unamended peat slurries, whether shaking or static. Although incubation of peat slurries provides homogeneous conditions and reduces environmental variables, the processing of the samples by diluting the peat soil, and shaking the incubations (Dannenberg et al., 1997) can perturb them (sometimes called the "vial effect") often releasing substrates or disrupting syntrophic interactions. The shift in non-amended samples towards members of the *Methanosarcinaceae*, metabolically versatile and relatively fast growing methanogens (Zinder, 1994), is consistent with greater substrate availability and suggests that the endogenous rates of methanogenesis in these samples are overestimates of those *in situ*.

Several studies have reported hydrogenotrophic methanogenesis as dominant in bogs while aceticlastic methanogenesis as important to dominant in minerotrophic fens (Avery et al., 1999, Popp et al., 1999, Chasar et al., 2000, Horn et al., 2003, Avery et al., 2003, Galand et al., 2005, Cadillo-Quiroz et al., 2006, Keller & Bridgham, 2007). In minerotrophic fens, the relative contribution of hydrogenotrophic methanogenesis has been found to range in the order of 30 to 55 % (Galand et al., 2005, Keller & Bridgham, 2007). Thus aceticlastic and hydrogenotrophic methanogenesis can be both significantly important in minerotrophic fens such as Michigan Hollow fen. The importance of both methanogenic pathways in Michigan Hollow is supported by the abundance of the two dominant members of the euryarchaeal community: MT, presumably aceticlastic methanogens, and E1, presumably hydrogenotrophic methanogens (Fig. 3-1 and 3-4).

All described *Methanosaeta* isolates are known to use only acetate for CH₄ production (Garcia et al., 2000), and have a lower minimum threshold for acetate (7 to 70 uM) than the other known aceticlastic methanogenic genus, *Methanosarcina* (0.2 to 1.2 mM) (Jetten et al., 1992). *Methanosaeta* can outcompete *Methanosarcina* in sites with low acetate concentration such as some minerotrophic fens (Galand et al., 2005), which is in agreement with the clone library and T-RFLP results observed in MH. MT was significantly more abundant than MS in the amplified portion of the archaeal community (Fig. 3-2 and 3-4) as similarly observed in a Finnish fen (Galand et al., 2005). The *Methanosaeta* clusters observed in this study (with a 97.5% similarity threshold) were similarly abundant in the different libraries but only one cluster (MT a in Fig. 3-1 and 3-2) had associated isolates.

The E1 group was recently identified by phylogenetic clustering and a common terminal restriction site in our T-RFLP analyses (Cadillo-Quiroz et al., 2006), and represented a minor fraction of methanogenic community in nearby bogs. The results in MH suggest that this group can be numerically significant and diverse in Michigan Hollow fen as indicated by its abundance in clone libraries, T-RFLP profiles, and existence of several sub clusters when applying a 97.5 % similarity cut off to clone libraries (Fig. 3-1, 3-2 and 3-4). In addition, sequences phylogenetically associated with E1 have also been detected in bogs, tundra wetland soil, anaerobic bioreactors, and landfill sites (Fig. 3-1), suggesting a broad ecological and geographical distribution of its members.

Group E1 belongs to the *Methanomicrobiales* order, does not have a reported isolated representative, and its closest cultured relatives are the hydrogenotrophic methanogens *Methanospirillum hungatei* and '*Candidatus* Methanoregula boonei' (Fig. 3-1). "*M. boonei*" is the first culture representative of the group E2 and was isolated from an acidic bog using low-ionic-strength media (Bräuer et al., 2006a,

Bräuer et al., 2006b). E1 was likely to be made up of hydrogenotrophic methanogens as indicated by the increase in the E1 peak in T-RFLP traces from H₂-CO₂-amended peat slurries (Fig. 3-5.B.3). However, *Methanospirillum* spp., a relatively fast-growing H₂-CO₂ utilizing methanogen which was poorly represented in MH clone libraries, commonly outgrew E1 in MH enrichments. Fortunately, the use of T-RFLP analysis allowed overcoming this interference by identifying a sample, among several treatments, where a member of the group E1 was preferentially enriched (Fig. 3-5.C.1). Several dilutions to extinction were subsequently performed and the selective enrichment of E1 verified by T-RFLP (Fig. 5.C). Strain E1-9c was obtained by this process and the purity of the isolate was tested and confirmed by several analyses (Fig. 3-6).

E1-9c is a novel isolate in the *Methanomicrobiales* order. It is associated to the group E1, being closest to members of the subgroup E1-c with 95 % similarity (Fig. 3-1). Group E1-c made up a small fraction in our libraries (Fig. 3-2) but, given that some sequences within this group showed a similarity lower than 97.5%, this group could contain other clusters which can be resolved as more sequences would become available in the future. In addition, very recently the isolation of strain NOBI-1 was reported (Sakai et al., 2007), but no information other than a 16S rRNA gene sequence was given. The NOBI-1 16S rRNA gene sequence has 94% identity with that from E1-9c and the predicted terminal restriction pattern for its rRNA gene places NOBI-1 in the E1 group, although its relationship with E1-9c and other members of E1 is unclear because of poor resolution in the part of the tree where it is located (Fig. 3-1). In terms of uncultured phylotypes, the closest relatives to E1-9c were environmental sequences recovered from an anaerobic bioreactors and landfills (Figure 3-1).

Strain E1-9c is a mesophilic and mildly acidiphilic methanogen (optimum growth at 30°C and pH 5.5). Interestingly, this strain required the presence of H₂S or

Na₂S but in concentrations below 0.1 mM (Figure 3-7.C), far lower than the 1-2 mM commonly added as a reducing agent in growth media for the culture of many other methanogens (Sowers & Noll, 1995, Ma et al., 2005, Kendall et al., 2006). H₂S and Na₂S have shown toxicity in anaerobic cultures (Brock & O'Dea, 1977, Mariotto et al., 1989) with a wide variability among methanogenic strains in terms of optimal and inhibitory concentrations (Jarrel & Kalmokoff, 1988). Na₂S toxicity in anaerobic media can be overcome by the addition of alternative reducing agents such as titanium (III) citrate or ferrous sulfide (Brock & O'Dea, 1977, Jones & Pickard, 1980) and minimal amounts of Na₂S to satisfy the sulfur requirements for growth reported to be in the order of 0.02 to 0.03 mM for some methanogenic strains (Rajagopal & Daniels, 1986). '*Candidatus* Methanoregula boonei' strain 6A8 is also inhibited by sulfide (Bräuer et al., 2006b), and it is likely that high sulfide concentrations in many standard growth media for methanogens are inhibitory to some presently uncultured methanogens.

Based on its phylogenetic and physiological characteristics, we propose E1-9c as the first representative of a new genus within the order *Methanomicrobiales*, under the name of '*Methanosphaerula palustris*' (Me.tha.no.spha.e'ru.la. N.Gr. n. methane; L. fem. n. sphaerula, a small sphere; N.L. fem. n. *Methanosphaerula* a spherically-shaped methane-producing Archaea; pa.lus'tris. L. fem. adj. palustris, palustre, pertaining to marshy, or swampy, living in marshes). Strain E1-9c have deposited in the corresponding culture collections (ATCC=BAA1556, DSM=19958) and an extended taxonomic characterization of this organism is in submission to the appropriate journal.

In summary, 16S rRNA gene-based methods were used to characterize the methanoarchaeal community in a neutral pH minerotrophic fen in upstate NY. The finding of many novel uncultured organisms, particularly in the E1 group, stimulated

our culture attempts. By coupling T-RFLP analysis, a quick tracking molecular tool with culturing techniques, we were able to isolate a novel methanogen in the *Methanomicrobiales* order. The E1 group and the E1-9c isolate have relatives inhabiting different habitats, including bioreactors, landfills, and peatlands, suggesting the importance of this relatively uncharacterized group in global methanogenesis.

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CHAPTER IV

***METHANOSPHAERULA PALUSTRIS* GEN. NOV., SP. NOV., A HYDROGENOTROPHIC METHANOGEN ISOLATED FROM A MINEROTROPHIC FEN PEATLAND.³**

4.1 ABSTRACT

Peatlands are important in CH₄ emissions to the atmosphere, and molecular surveys have identified a diverse but mainly-uncultured euryarchaeal community in them. Here we report the characterization of a strain, E1-9c^T, associated with the uncultured group E1 from a minerotrophic fen. Cells were regular cocci, usually in pairs, stained Gram negative, and were resistant to 0.1% SDS lysis. Multiple flagella were observed but motility was not observed in wet mounts. The isolate grew at mesophilic (28-30°C) and slightly acidophilic (pH 5.5) as its optimal conditions. It had a low Na⁺ and NaCl tolerance (<100mM and <0.375% respectively) and was sensitive to Na₂S concentrations above 0.1mM. H₂/CO₂ and formate are the only methanogenic substrates used by E1-9c^T; and formate concentrations above 50 mM were inhibitory for growth. In addition to methanogenic substrates and mineral salts, it required vitamins, coenzyme M, and acetate (4mM) for growth, with a doubling time of near 19 h. The phylogenetic analysis of the 16S rRNA and inferred McrA amino acid sequences showed E1-9c^T as an independent group within the *Methanomicrobiales* order. The physiological and phylogenetic comparisons with different members from the order support the classification of E1-9c^T as a new genus in the *Methanomicrobiales*. We propose the name *Methanosphaerula palustris* gen. nov., sp. nov., with its type strain E1-9c^T (ATCC=BAA-1565, DSM=19958)

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4.2 INTRODUCTION

Peat-forming wetlands or peatlands (Gore, 1983) store nearly 30% of all terrestrial carbon (Gorham, 1991) and are estimated to contribute around 20% (115Tg) of total annual CH₄ emissions to the atmosphere (Cicerone & Oremland, 1988). Hence, the methanogenic archaea inhabiting these ecosystems are of importance, particularly since the CH₄ concentration in the atmosphere has more than doubled in the past 200 years (Cicerone & Oremland, 1988). 16S rRNA gene surveys from acidic and neutral pH peatlands have shown a broad diversity of euryarchaeal sequences (Cadillo-Quiroz et al., 2008, Cadillo-Quiroz et al., 2006, Galand et al., 2003, Kotsyurbenko et al., 2004). The majority of sequences were not closely related to cultured strains but instead formed independent clusters, suggesting the existence of novel euryarchaeal groups. Two of these clusters, named “E1” and “E2” groups in the *Methanomicrobiales* (Cadillo-Quiroz et al., 2006), have been shown to be highly abundant in clone libraries and terminal restriction fragment length polymorphism (T-RFLP) analysis from poor acidic bogs and near-neutral pH fen peatlands (Basiliko et al., 2003, Cadillo-Quiroz et al., 2008, Cadillo-Quiroz et al., 2006).

Several phylogenetic analyses have shown that E1 and E2-related sequences form independent branches within the *Methanomicrobiales* (Basiliko et al., 2003, Cadillo-Quiroz et al., 2008, Cadillo-Quiroz et al., 2006, Galand et al., 2003, Hales et al., 1996). The order *Methanomicrobiales* is a diverse group and its members are considered to exhibit the greatest variability in morphology and physiology amongst methanogenic archaea (Dubach & Bachofen, 1985). This order is comprised of the families *Methanomicrobiaceae*, *Methanocorpusculaceae* and *Methanospirillaceae* (Boone et al., 2001, Garcia et al., 2000). It also includes two genera that are not placed within any family: the genus *Methanocalculus* which is listed as *genus incertae*

(Boone et al., 2001) with 3 reported species (Lai et al., 2004, Mori et al., 2000, Ollivier et al., 1998), and the proposed genus *Methanoregula* with *M. boonei* as its representative strain isolated from an acidic bog (Bräuer et al., 2006b, Bräuer et al., 2006a).

M. boonei is a member of the E2 group and was isolated using low ionic strength media and reducing agents other than Na₂S to avoid observed methanogenic growth inhibition (Bräuer et al., 2006b). Recently, we have reported the isolation of a novel methanogen designated strain E1-9c^T using the same culturing medium coupled to molecular analysis as a selection tool (Cadillo-Quiroz et al., 2008). Strain E1-9c^T was isolated from a minerotrophic fen in upstate New York (42° 21' N, 76° 28' W) where sequences belonging to the E1 group were diverse and abundant (Cadillo-Quiroz et al., 2008). In our previous paper, we reported that after using T-RFLP analysis as a selection and tracking tool, we were able to find conditions that enriched and allowed for the isolation of a member of E1. We evaluated the culture purity by culture techniques, phase contrast microscopy, fluorescent *in situ* hybridization (FISH), T-RFLP and clone library analysis, and proposed E1-9c as the first member of a novel genus within the *Methanomicrobiales*. In this report we present a more detailed evaluation of the morphological, physiological and phylogenetic characteristics of strain E1-9c^T. We describe the new genus and species *Methanosphaerula palustris* gen. nov. sp. nov with strain E1-9c^T as its representative.

A modified version of the PM1 medium (Bräuer et al., 2006b) named PM2 was used in this study with the following components (in mg L⁻¹): 1.5 KCl, 13.6 KH₂PO₄, 26.8 NH₄Cl, 0.024 CoCl₂·6H₂O, 0.075 ZnCl₂, 0.019 H₃BO₃, 0.024 NiCl₂·6H₂O, 0.024 Na₂MoO₄·2H₂O, 1.3 FeCl₂·4H₂O, 0.026 MnSO₄·4H₂O, 1.6 MgSO₄, 2.4 CaCl₂·2H₂O, 0.009 CuSO₄·5H₂O, 3.5 AlK(SO₄)₂·12H₂O and 3.7 of disodium EDTA. Medium was prepared under a 70%N₂/30%CO₂ atmosphere as described previously

(Bräuer et al., 2006b) using the modified anaerobic technique of Hungate (Sowers & Noll, 1995). Several hours before inoculation, sterile anaerobic additions were made to the basal medium with the following final concentrations: 1.0 mM titanium (III) nitrilotriacetate (7.2 mL 1M Tris-HCl adjusted to pH 8, 4.8 mL of 0.5 M sodium nitrilotriacetate and 0.55 mL of 15% titanium III chloride from Fluka), 10 mM MES (2-(N-morpholino) ethane sulfonic acid, $pK_a = 6.2$ at 28 °C, filter-sterilized 1 M stock solution adjusted to pH 7.5), 0.5 mM coenzyme-M (2-mercapthoethanesulfonic acid), 0.4 mM sodium acetate, 1% v/v vitamin solution (Balch et al., 1979) and 0.04 mM Na_2Sx9H_2O (filter-sterilized 20 mM stock solution). The final liquid volumes in the tubes were ca. 5 ml, and 70.7 kPa H_2/CO_2 (80%/20%) was added to the headspaces unless otherwise specified when testing other methanogenic substrates. The cultures were incubated on a gyratory shaker at 30°C and 200 rpm and all evaluations were done for over 29 days.

Cell morphology and motility were examined under phase contrast microscopy with a Nikon Eclipse E600 microscope. Gram staining and susceptibility test to DSD lysis were done as previously described by Boone and Whitman (1988). SDS tests were done with the final concentrations of 0.1% to 5%, and cell lysis was determined by microscopic observations. Negative staining and ultra-thin sectioning transmission electron microscopy was performed as described elsewhere (Firtel et al., 1995) using 2% uranyl acetate (pH 6.5) and a Phillips Technai 12 Biotwin electron microscope equipped with a Gatan Multiscan model 791 camera and Digital Micrograph software. Photos were taken at 100 KV. Flagella were observed with low uranyl acetate staining (0.2%) and for visual purposes images were digitally enhanced, using the Photoshop software, by a differential adjustment of the brightness and contrast of cell body and flagella versus background surface.

To determine the effect of sodium on the growth of strain E1-9c^T, sterile additions from anaerobic NaCl stocks of different concentrations were made to achieve values between 36 to 270 mM. Background sodium content (ca. 15 mM), from the different components of the medium, was added to NaCl values. Substrate utilization tests were done in duplicate with substrates added to both a set of tubes under N₂/CO₂ atmosphere and another set under H₂/CO₂ atmosphere, the former was done to verify that the substrate was non-inhibitory. The following substrates were tested at a 10 mM final concentration: sodium acetate, trimethylamine, propanol, methanol, ethanol, 2-butanol, sodium propionate, sodium butyrate, and sodium formate. The formate utilization tests also included treatments with or without the addition of sodium selenite (5uM) to the media, and similar additions but at a higher pH (6.4). The effect of acetate as a carbon source was tested by incubating tubes under H₂/CO₂ atmosphere with the addition of sodium acetate to 0.4, 4, 20 and 70 mM final concentration. Antibiotic susceptibility tests were done by adding anaerobic filter-sterilized stocks of penicillin, ampicillin, vancomycin, tetracycline, bacitracin, kanamycin, spectinomycin, rifampicin and chloramphenicol (each to 100 ug mL⁻¹ final concentration). Both sodium and antibiotic sensitivity tests were done under a H₂/CO₂ atmosphere for over a month. Treatments were monitored for CH₄ production with a flame ionization gas detector as described previously (Cadillo-Quiroz et al., 2006). Growth rates were calculated from CH₄ production from the exponential parts of the CH₄ accumulation curve (Ni & Boone, 1991).

E1-9c^T's 16S rRNA amplification, cloning and sequencing have been described in a previous report (Cadillo-Quiroz et al., 2008). *McrA* gene amplification used the ME1 and ME2 primer set with similar conditions as described previously (Hales et al., 1996). Phylogenetic analyses of 16S rRNA gene sequences used the ARB software (Ludwig et al., 2004) and the latest release (January, 2007) of the

“greengenes” database (DeSantis et al., 2006). The 16S rRNA gene sequence alignment was exported from ARB using an “Archaeal” nucleotide base frequency filter that included positions with more than 50% invariance (1140 valid columns) to avoid possible treeing artifacts. Phylogenetic trees were constructed by Bayesian analyses. The approximation of posterior probabilities was done with MrBayes version 3.0 (Ronquist & Huelsenbeck, 2003) using four-chain Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) analysis. Trees and model parameter values were sampled from a target distribution generated when chains converged. Bayesian consensus trees were built with a burnout of 300 and posterior probabilities were calculated. Tree topology was confirmed using maximum likelihood and neighbor joining methods (implemented in ARB) with Olsen evolutionary distance correction. The presented tree represent the most frequently observed topology across various phylogenetic analyses. For *McrA*-predicted amino acid sequences, the sequences were aligned using CLUSTALX , and analyzed using MrBayes with similar conditions as described above. 236 amino acid residues were compared for the *McrA* tree.

The G+C content of E1-9c^T’s genomic DNA was calculated with a thermal denaturation fluorimetric method using the Bio-Rad iCycler iQ real time PCR system (Bio-Rad Laboratories) as described previously (Gonzalez & Saiz-Jimenez, 2002). The G-nome kit (Qbiogene), following the manufacturer’s instructions, was used to isolate genomic DNA from the strain E1-9c^T and the following standards: *Methanoregula boonei* 6A8 (54.5 mol% G+C, S. Brauer personal communication), *Escherichia coli* K-12 (50 mol% G+C) and *Pseudomonas aeruginosa* PAO1 (66 mol% G+C). In addition, the DNA of *Micrococcus luteus* (72 mol% G+C) and *Clostridium perfringens* (27 mol% G+C) were also used as standards and obtained from commercial sources (Sigma-Aldrich). Thermal denaturation was performed with approximately 2.5 µg DNA from isolate and standards. Thermal conditions consisted

of a ramp from 55 °C to 90 °C at 0.5 °C min⁻¹. Fluorescent DNA melting curves were generated in triplicate. The DNA G+C content for strain E1-9c^T was calculated using a linear regression analyses of melting temperatures (T_m) against the G+C content of the standards' DNA.

4.3 RESULTS AND DISCUSSION

The E1-9c^T cells were regular coccus-shaped, 0.5-0.8 µm in size and stained Gram-negative. The cells were mostly associated in pairs and planes of division were commonly visible (Fig. 4-1A). Other coccus-shaped organisms in the *Methanomicrobiales*, such as *Methanogenium*, tend to be irregular and do not form pairs. *Methanofollis aquaemaris* N2F9704^T forms regular cocci when in exponential phase but changes to irregular cocci in stationary phase (Lai & Chen, 2001), E1-9c^T cultures did not show such a change in morphology. Under phase-contrast microscopy E1-9c^T cells appeared with a dark centre surrounded by a transparent outer layer; and the cells were autofluorescent when exposed to near UV light cells indicating the presence of Factor₄₂₀, a fluorescent coenzyme often present in high concentrations in methanogens (Garcia, 1990). The cell envelope was resistant to 0.1% SDS lysis although concentrations above 2% showed some evidence of envelope damage.

Transmission electron microscopy (TEM) of negatively stained cells (Fig. 4-1B), showed that cells are multi-flagellated. E1-9c^T flagella were around 14 nm thick and could reach lengths of 8-12 µm. Flagella were readily lost under stress conditions such as high speed centrifugation and oxic environment, perhaps explaining the lack of motility observed in light microscopy of wet mounts. In thin sections, the cell envelope is about 30-70 nm thick and electron dense, resembling pseudomurein which is common in members of the *Methanobacteriales* but has not been found in *Methanomicrobiales* (Sprott & Beveridge, 1994).

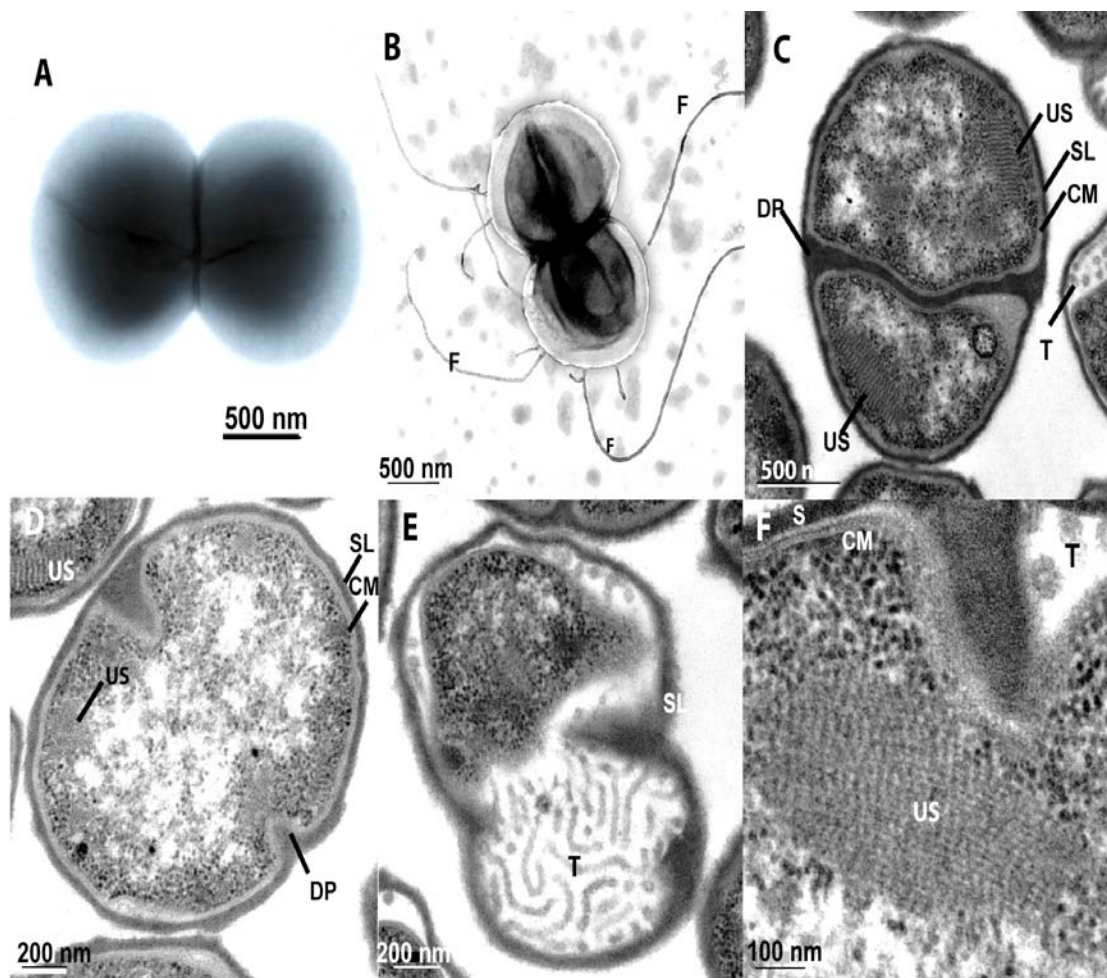


Figure 4-1. Transmission electron microscopy of E1-9c^T cells: A) negative stain showing the regular coccus morphology, B) negative staining with low uranyl acetate addition and digital enhancement (see above) to allow contrast for the observation of multiple flagella (F), C-F) ultra-thin sectioning microscopy showing several intracellular details as S-layer (SL), cytoplasmic membrane (CM), cell's division plane (DP), tubular-like S-Layer-connecting structures (T) and unknown intracellular structure (US).

In some thin sections, some cells were apparently shrunk away from the outer layer by the osmotic strength of the 2% glutaraldehyde fixative, tubular structures were visible (T in Fig. 4-1 D-F). These tubular structures have been suggested to connect the S-layer to the cytoplasmic membrane (Sprott & Beveridge, 1994) and are identical to those observed in *Methanosarcina mazei* (Robinson, 1986). Additionally, in many cells an unknown structure was observed in the cytoplasm (US in Fig. 4-1C, D and F)

resembling arrays of protein complexes ca. 10 nm in diameter that were less electron-dense than ribosomes. These structures do not resemble the "methanochondrion" internal membrane systems now believed to be preparation artifacts (Sprott & Beveridge, 1994). The potential protein complexes are similar in size to purified methylcoenzyme M methylreductase complexes (ca. 10 nm) and smaller than F₄₂₀-reducing hydrogenase complexes (ca. 15 nm) observed by electron microscopy (Wackett et al., 1987).

Our previous report (Cadillo-Quiroz et al., 2008) showed that E1-9c^T is a mesophile with optimal growth at 28-30°C and is mildly acidophilic with an optimum pH near 5.5 and a narrow range from 4.8 to 6.4. Only a few other methanogens have been found to have a pH optimum near 5.5 or below and only a newly isolated strain, *Candidatus Methanoregula boonei* 6A8, belongs to the order *Methanomicrobiales*. *M. boonei* is the most acidiphilic methanogen described to date with an optimal pH near 5.1 and was isolated from an acidic (pH 4.5) bog peatland (Bräuer et al., 2006a). *Methanobacterium espanolae*, from the *Methanobacteriales* order (Garcia et al., 2000), represent the next most acidiphilic reported methanogen with an optimum pH near 5.6 and was isolated from pulp mill waste sludge (Patel et al., 1990). Strain E1-9c^T has a lower optimum pH than that of the fen from which it was isolated (pH 6.5).

The optimum Na⁺ concentration for the strain E1-9c^T was between 15 (background Na⁺ concentration in medium) and 35 mM, and concentrations above 100 mM inhibited growth (Fig. 4-2). The sodium chloride tolerance range was from 0 to 0.35%, which a value lower than many strains isolated from other non-marine sources (Table 4-1).

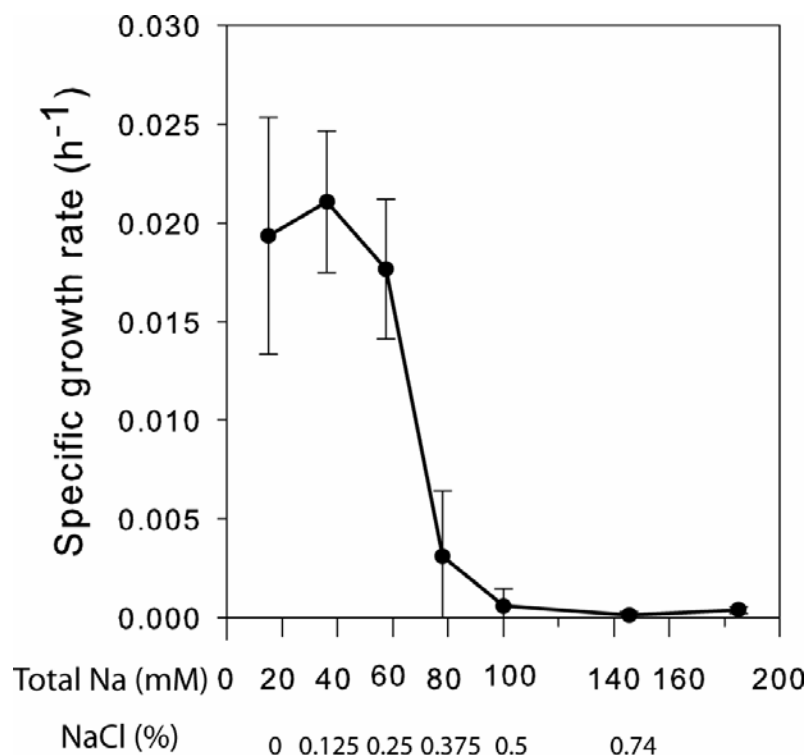


Figure 4-2. Na effect on E1-9c^T's growth. Total Na includes background Na in media plus additions from NaCl. N=3 ± SD.

The inhibitory concentration of Na⁺ in E1-9c^T was near double the amount than the one for *M. boonei* (S. Brauer, personal communication). The difference in Na⁺ tolerance between E1-9c^T and *M. boonei* could hypothetically represent an adaptation to the difference in Na⁺ concentration observed in the pore water of Michigan Hollow (ca. 30 μM Na⁺) versus McLean Bog (ca. 2 μM Na⁺) (Dettling et al., 2007). E1-9c^T was also highly sensitive to Na₂S concentrations. E1-9c^T requires the presence of Na₂S as used by methanogens as a sulfur source (Rajagopal & Daniels, 1986), but concentrations above 0.1 mM are inhibitory to its growth (Cadillo-Quiroz et al., 2008). Na₂S is commonly use as a reducing agent in liquid media at concentrations between 1-2 mol L⁻¹ (Sowers & Noll, 1995).

In terms of methanogenic substrates, E1-9c^T cultures used H₂/CO₂ but not acetate, trimethylamine, propanol, methanol, ethanol, 2-butanol, propionate or sodium butyrate (all at 10 mM final concentration) under standard growth conditions. With the exception of formate, none of these substrates had inhibitory effects on cultures grown with H₂/CO₂. Formate additions (10 or 20 mM) caused a three to four week lag for growth in tubes also supplemented with H₂/CO₂ (Fig. 4-3).

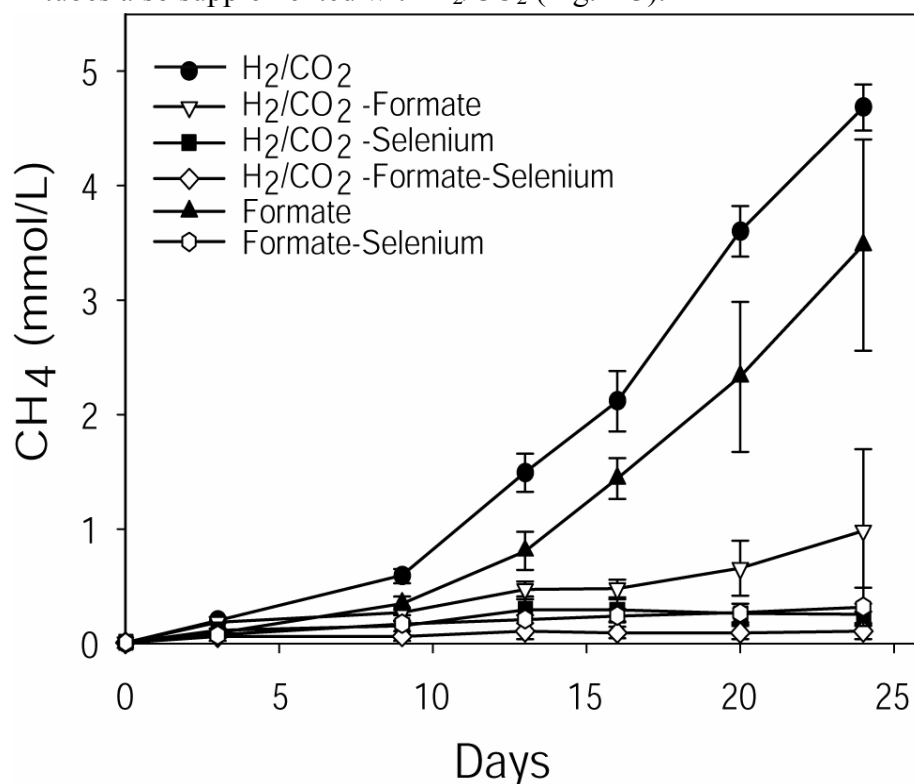


Figure 4-3. E1-9c^T's formate utilization tests. Multiple treatments were done with the addition of 70.7 kPa H₂/CO₂ (80%/20%), 20 mM sodium formate and 1 uM sodium selenite final concentration as indicated in graph legend. N=3 ± SD.

Formate is commonly used amongst members of the order *Methanomicrobiales* (Boone et al., 2001); however, reports have shown that in some strains formate can be toxic at concentrations ≥ 100 mM at low pH (<6) or that the activity of the formate dehydrogenase is dependent on the presence of selenium (Belay et al., 1986, Jarrel & Kalmokoff, 1988, Sparling & Daniels, 1990). Therefore, we performed additional experiments to address the potential formate toxicity at low pH

or selenium requirement. At pH 5.7, strain E1-9c^T was able to use formate when it was kept at low concentration by multiple 10-20 mM additions following the stoichiometric increase of CH₄ (Fig. 4-3). Formate additions above 50 mM inhibited E1-9c^T growth. Higher pH (6.4) or the addition selenite (1 and 10 μM), did not improve the use of formate by E1-9c^T (Fig. 4-3). The addition of 1 μM selenite accentuated the inhibitory effects of formate in tubes with H₂/CO₂ (Fig. 4-3).

In addition to H₂/CO₂ as methanogenic substrate and mineral nutrients, E1-9c^T required vitamins, coenzyme M, and acetate in the medium. The use of acetate as a carbon source has been observed in many H₂/CO₂-utilizing methanogens (Balch et al., 1979, Belay et al., 1986, Bott et al., 1985, Jarrell & Kalmokoff, 1988). For instance, *Methanospirillum hungatei* requires acetate at levels around 18 mM (Sprott & Jarrell, 1981), and in *Methanobacterium thermoautotrophicum* acetate was measured to contribute up to 65% of total cellular carbon (Fuchs et al., 1978). We found that concentrations around 4 mM acetate were the optimal for methanogenic growth under H₂/CO₂, and that concentrations above 20 mM did not further stimulate E1-9c^T's growth (data not presented). E1-9c doubling time was ca. 30 hours with 0.4 mM (concentration used in the isolation of the strain) and 19 hours with 4mM acetate under standard growth conditions (pH 5.7 and 30°C).

Antibiotic susceptibility tests showed that strain E1-9c^T was resistant to penicillin, ampicillin, vancomycin, tetracycline, bacitracin, kanamycin, spectinomycin and rifampicin (100 μg mL⁻¹), but chloramphenicol inhibited its growth.

The full 16S rRNA gene and intergenic transcribed sequence (ITS) were sequenced for strain E1-9c^T and the phylogenetic analysis showed this strain was closely associated to the environmental sequences sub cluster “c” of the E1 group (Cadillo-Quiroz et al., 2008). The E1 group is located as an independent branch within the order *Methanomicrobiales* (Cadillo-Quiroz et al., 2008, Cadillo-Quiroz et al.,

2006). Therefore, we constructed a 16S rRNA and *mcrA* based phylogenetic trees (Fig 4-4) with predicted sequences of representative strains of the reported genera in this order. We also included the newly proposed methanogen genus *Methanoregula* –strain *M. boonei* 6A8 (Bräuer et al., 2006a), and the newly reported strain NOBI-I (Sakai et al., 2007).

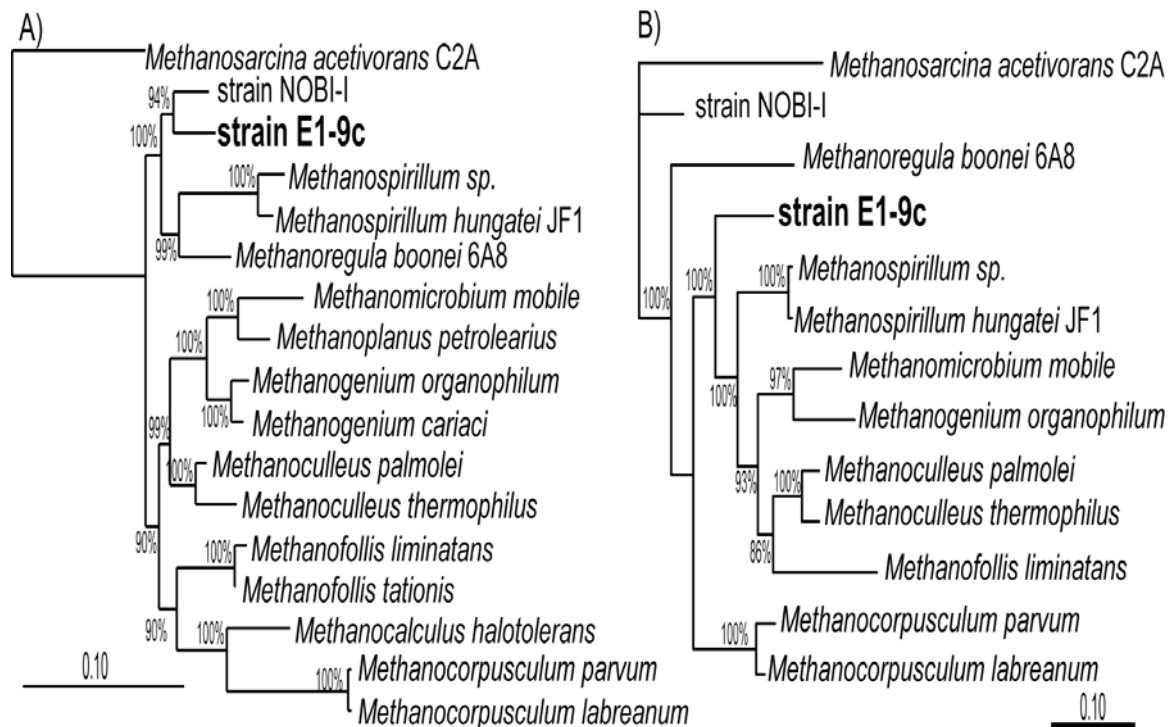


Figure 4-4. Phylogenetic analysis of strain E1-9c^T and members of the order *Methanomicrobiales* based on 16S rRNA gene (A) and *Mcr-A* (B) amino acid sequences. The accession numbers of 16S rRNA and *mcrA* gene sequences used in the analysis are as follows: *M. acetivorans* (NC_003552, AAMO7885), NOBI-I (AB162774, BAF56441), *M. boonei* (DQ282124, ABB53477), *Methanospirillum* sp. (AJ133792), *M. hungatei* (CP000254, AAK16835), *M. thermophilus* (M59129, AAK16834), *M. palmolei* (Y16382, BAF56663), *M. cariaci* (M59130), *M. organophilum* (M59131, BAF74593), *M. mobile* (M59142, AAL29293), *M. liminatans* (Y16429, AAL29290), *M. tationis* (AF095272), *M. parvum* (AY057068, AAP20900), *M. labreanum* (AY260436, ABN07725), *M. taiwanensis* (AF172443), and *M. halotolerans* (AF033672).

The 16S rRNA phylogenetic analysis showed that E1-9c^T is divergent from all other described genera and families within the *Methanomicrobiales* with only 85-92% identity to any of them. The phylogenetic branching order in Figure 4-4 indicates that

Methanospirillum, and hence the *Methanospirillaceae* family, is the most closely related group (90% identity). However, in terms of sequence identity, the closest relatives of strain E1-9c^T were strain NOBI-I (94% identity) followed by *Methanoregula boonei* 6A8^T and *Methanoculleus palmolei* ISNLUZ^T (both with 92% identity). The phylogenetic analysis of the *mcrA* gene supported strain E1-9c^T as an independent branch in the *Methanomicrobiales*, although when compared to the 16S rRNA gene analysis, some differences are observed in the branching order and order of sequence similarity with its closest relatives (Fig. 4-4). *McrA* sequence analysis showed the following order in terms of the percent amino acid sequence identity to E1-9c^T: *M. palmolei* ISNLUZ^T (83%) as the closest, followed by *M. hungatei* (82%), and *M. boonei* 6A8 and NOBI-I (both 81%). Contrary to the 16S rRNA gene analysis, NOBI-I was more distantly related to E1-9c^T; however the branching position of NOBI-I, E1-9c, and 6A8 were highly variable in the multiple analyses of *mcrA* gene predicted aminoacid sequences indicating that these sequences did not provide a robust phylogeny for these organisms. Strain NOBI-I was isolated using a H₂-syntrophic enrichment technique (Sakai et al., 2007), but no information other than a 16S rRNA and *mcrA* gene sequences was reported when preparing this manuscript which precluded us from further comparisons. The analysis of the G+C molecular content indicated that E1-9c^T contains around 58.9 ±2 mol %.

Table 4-1. Physiological characteristics and comparison of strain E1-9c^T with other representative coccoid and close phylogenetic relative methanogens belonging to the order *Methanomicrobiales*

Species	Source	Cell shape	pH optimum & range	Temp optimum & range (°C)	NaCl optimum & range (%)	Minimum generation time, (hr ⁻¹)	G+C content (mol %)	Substrates used				AR
								H	F	2P	2B	
Strain E1-9c*	Rice fen	Regular cocci	5.7 4.3-6.4	38 14-35	<0.125 <0.375	19	58.9	+	+	-	-	+
<i>Methanoregula boonei</i> [¶]	Acidic bog	Thin rod & irregular cocci	5.1 4.5-5.55	35-37 10-40	ND	44	54.5	+	-	-	-	+
<i>Methanospirillum hungatei</i> [§]	Sewage sludge	spirillum	6.6-7.4 ND	30-37 ND	ND	17	45	+	+	ND	ND	+
<i>Methanococcus paludis</i> [¶]	Anaerobic digester	Irregular cocci	6.9-7.5 6.3-8	40 22-50	ND	13.5	59	+	+	+	+	ND
<i>Methanofollis capemaris</i> [*]	Fish pond	Regular and irregular cocci	6.5 6.3-8	37 20-43	0.5 0-6%	63	ND	+	+	-	-	-
<i>Methanogeeva aggregans</i> [?]	Anaerobic digester	Irregular cocci	6.5-7 6-8	37 25-40	<0.2% <0.2%	8	52	+	+	ND	ND	+
<i>Methanoplanus petroleocrinus</i> [‡]	Oil well	Irregular cocci	7 5.3-8.2	37 28-43	1-3 0-5	10	50	+	+	+	ND	+
<i>Methanococcus parvus</i> [‡]	Anaerobic digester	Irregular cocci	6.8-7.5 ND	37 25-44	0-4.7 ND	8	49	+	+	+	+	+
<i>Methanococcus luteus</i> [‡]	Oil well	Irregular cocci	7.6 7-8.4	38 25-45	5 0-12.5	12	55	+	+	-	-	+

*:This study, ¶: strain 6A8 (Bräuer et al., 2006a), §: strain JF1^T (Balch et al., 1979, Ferry et al., 1974), ψ: strain INSLUZ^T (Zellner et al., 1998), Φ: strain N2F9704^T (Lai & Chen, 2001), γ: strain MST^T (Ollivier et al., 1985), ε: strain SEBR 4847^T (Ollivier et al., 1997), ζ: strain XII^T (Zellner et al., 1987), £: strain SEBR 4845^T (Ollivier et al., 1998)

H= H₂/CO₂; F= formate; 2P= 2-propanol; 2B= 2-butanol; ND= not determined; AR=acetate requirement for growth

The comparison of phenotypic (Table 4-1) and phylogenetic (Fig. 4-4) characteristics of strain E1-9c^T indicates its uniqueness among members of the order *Methanomicrobiales*, allowing us its proposal as the type species of a new genus, *Methanosphaerula palustris* gen. nov., sp. nov. We proposed this genus as a *Genus incertae sedis* (GIS) temporary not affiliated to any of the known families in this order. In the 16S rRNA gene phylogenetic analysis within the *Methanomicrobiales*,

the order of phylogenetic divergence of E1-9c with members of the other families (85-92% identity) was close to the divergence levels observed among the families themselves (82-89%), which suggests that E1-9c^T could represent a new family. We expect that a more detailed genomic analysis of this strain presently underway and the isolation and description of additional related strains (such as NOBI-I) will allow a better evaluation of the higher-order taxonomy of these novel organisms within the *Methanomicrobiales*.

Description of *Methanosphaerula* gen. nov.

Methanosphaerula (Me.tha.no.spha.e'ru.la. N.Gr. n. methane (from N.Gr. n. meth (yl) and chemical suffix -ane), methane; L. fem. n. sphaerula, a small sphere; N.L. fem. n. *Methanosphaerula* a spherically-shaped methane-producing *Archaea*).

Cells are coccus-shaped often in pairs. Methanogenic and strictly-anaerobic members of the domain *Archaea*. Mesophilic and mildly acidophilic; produces CH₄ from H₂/CO₂ and formate. The type species is *Methanosphaerula palustris*.

Description of *Methanosphaerula palustris* sp. nov.

Methanosphaerula palustris (pa.lus'tris. L. fem. adj. palustris, paluster, palustre, pertaining to marshy, swampy or muddy, living in marshes)

Cells are coccus shaped and usually present as pairs, with Gram negative reaction and do not lyse with 0.1% SDS. Cells are 0.5-0.8 µm in diameter and are autofluorescent when exposed to near UV light. The optimum pH is near 5.5; growth occurs from pH 4.8-6.4. The temperature optimum is near 30°C with growth occurring from 14 to 35°C. Growth is optimum from 15 to 35 mM Na⁺ but no growth is observed in the presence of ≥ 100 mM Na⁺/0.375% NaCl. Doubling time is about 19 h under optimal conditions. CH₄ is produced from H₂/CO₂ or formate, but not ethanol,

methanol, 2-isopropanol, 2-isobutanol, acetate, propionate, or butyrate. Formate concentrations >50 mM are inhibitory at pH 5.5. Vitamins, coenzyme M, acetate (4 mM) and low concentrations of Na₂S (< 0.08mM) are required for growth. The DNA G+C content is ~58.9 %. The isolate was derived from minerotrophic fen peatland. The type strain is E1-9c^T, and is deposited in ATCC and DSM (BAA-1565 =19958)

4.4 ACKNOWLEDGEMENTS

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CHAPTER V

**MODELING OF ENVIRONMENTAL INFLUENCES OVER GENE
SEQUENCES TO ASSESS MICRODIVERSITY PATTERNS OF ARCHAEAL
POPULATIONS**

5.1 ABSTRACT

Fine scale phylotype diversity, or gene sequence microdiversity, has been observed in molecular surveys of the 16S rRNA gene and the internally transcribed sequence (ITS) between 16S and 23S rRNA genes of microorganisms from terrestrial and marine ecosystems. We surveyed the 16S rRNA-ITS sequence diversity of the E2/*Methanoregula*, a group in the *Methanomicrobiales* known to be important in acidic bogs. The phylogenetic analysis of recovered sequences showed a contrasting gene microdiversity patterns in samples from two bogs with similar dominant vegetation (*Sphagnum* mosses), surface pH and low ion composition, but with different site developmental history. The populations in McLean Bog were more diverse than those in Chicago Bog. An individual based model (IBM) was implemented to explore the theoretical scenario that the contrasting phylogenetic patterns had an ecologically-driven origin due to the different developmental histories of the sites. The model simulated the population dynamics of phenotypically varying individuals from this group for 5500 generations under changing carrying capacity (K) and variance of competition (C). K and C were assumed to have different values for the three stages of site development (pond, fen and bog). To assess environmental effects on neutral gene sequences, a short vector (250 bases) was added to each individual with a low mutation rate.

The simulations showed that individual-environment and individual-individual interactions had an imprint on gene sequences as demonstrated by different tree

topologies. Simulation results indicated that differential microdiversity patterns due to different developmental histories were theoretically possible. Phylogenetic analysis of simulated gene sequences showed strikingly similar patterns to those from environmental sequences. This modeling could represent a unique tool for exploring alternative scenarios for gene diversity and analysis of lineages' numbers in a phylogenetic tree.

5.2 INTRODUCTION

It is likely that *Bacteria* and *Archaea* have existed on Earth for at least 3.8 billion years (DeLong & Pace, 2001). Not surprisingly, these groups exhibit a ubiquitous distribution around the globe (Boone et al., 1993, Rappe & Giovannoni, 2003) and are the most abundant organisms on Earth with an estimated number as high as $4\text{--}6 \times 10^{30}$ cells (Whitman et al., 1998). In addition, these groups are found to encompass a broad taxonomic, evolutionary and metabolic diversity unparalleled in the biological world (Horner-Devine et al., 2004). Empirical estimations of microbial species diversity, using an operative definition based on DNA re-association, found that as many as 6000 species (Torsvik et al., 2002) or as many as 10^7 (Gans et al., 2005) can exist in a gram of pristine forest soil. Statistically-based estimations place the value of $2\text{--}4 \times 10^6$ species as the likely limit for all microbial diversity (Curtis et al., 2002), although it should be pointed out that the definition of a prokaryotic species is subjective and generally much broader than that for eukaryotes. Despite the impressive amount of diversity, little is known about the origin, maintenance and distribution of microbial diversity since most studies have focused on plant and animal diversity (Horner-Devine et al., 2004).

Every organism interacts with its surrounding environment, and this interaction is a key factor for group success and survival. Ecologists often treat conspecific

individuals as ecologically equivalent, but individual variation has been found to be widespread (Bolnick et al., 2003), although of unpredictable nature. This individual variation is vertically inheritable, particularly in non-sexual populations such as microorganisms. Thus, individual variation provides the basis for differential response to environmental conditions, ecological specialization, and adaptive speciation. Genetic individual variation has been observed in cultured microbial strains from similar populations using high resolution molecular profiling techniques (Cho & Tiedje, 2000, Schlöter et al., 2000), or sequencing of housekeeping genes (Feil & Enright, 2004, Maiden et al., 1998). In the case of pathogenic isolates, this genetic variation correlated with phenotypic variation was related to pathogenic outbreaks (Chan et al., 2001). Although cultured microorganisms represent a small fraction of the microbiota of natural ecosystems, such as soil, individual variation seems to be ubiquitous. We could reasonably expect similar if not higher individual variation in the dominant non-cultured microbial fraction, since the uncultured proportion is commonly more numerous and broadly distributed.

Sympatric speciation, or the splitting of a lineage under conditions of ecological contact within the same spatial habitat (Turelli et al., 2001), has received significant empirical and theoretical support as a mechanism for the origin of diversity (Via, 2001). Particularly, the theory of adaptive dynamics has provided a mathematical framework for studying adaptive sympatric diversification (Metz et al., 1996). Under this theory, evolutionary branching is presented as a theoretical metaphor for adaptive splitting due to frequency-dependent individuals' interactions under ecological scenarios (Doebeli & Dieckmann, 2000) such as competition for resources (Dieckmann & Doebeli, 1999). Experimental evidence of adaptive diversification under sympatric conditions in microorganisms has been provided using *E. coli* populations and the theoretical analysis of the adaptive dynamics of sequential

use of different substrates (diauxic⁴ populations) (Friesen et al., 2004). This adaptive dynamics analysis showed that the evolutionary branching caused by diauxic behavior was theoretically possible, and the likely scenario for the origin of dimorphic *E. coli* populations (Friesen et al., 2004).

Adaptive dynamics simulates the ecological splitting of populations. However, currently it does not incorporate genetic and phyletic information or the effects of ecological interactions on them. Therefore, the possibility to use phylogenetic tests to evaluate ecological and evolutionary hypotheses (and vice versa) is not possible (Whelan et al., 2001). To explore the potential effects of ecological interactions and branching on microbial genetic diversification, approaches that relate ecological adaptation with genetic and phyletic diversification are required.

Phylogenetic approaches are commonly used in the attempt to reveal the evolutionary branching order of organisms at different phyletic levels (from domains to subpopulations). Fine scale gene “microdiversity” has been observed in environmental surveys of marker genes, such as 16S rRNA, in the non-cultured fraction of microorganisms of marine (Acinas et al., 2004) and terrestrial ecosystems (Schloter et al., 2000, Zhou et al., 2004). Microdiversity has been suggested to potentially originate from variability among microbial populations. In this study, cloning and sequencing of the 16S rRNA gene and the internally transcribed sequence (ITS) region between it and the 23S rRNA gene were done to target an archaeal group dominant in acidic bogs, the E2/*Methanoregula* group (Bräuer et al., 2006, Cadillo-Quiroz et al., 2006). Contrasting microdiversity patterns were found in samples from two acidic bogs with different developmental history. We hypothesized that the

Diauxic: describes bacterial growth as it metabolizes a mixture of substrates. Cells often preferentially metabolize one substrate before another, with the classic example being the preferential utilization of glucose over lactose by *Escherichia coli*.

contrasting microdiversity patterns have an ecological origin according to the ecological succession at each site. Adaptive dynamic simulations of individuals carrying evolutionary neutral gene sequences were done to test the theoretical possibility of this scenario.

5.3 METHODS

Study site and sampling

McLean Bog (MB) and Chicago Bog (CB) are located within 40 km of Ithaca NY (42° 30' N, 76° 30' W) in the northeast USA, and both sites have been described previously (Osvald, 1970). Briefly, MB is an ombrotrophic kettle hole bog approximately 0.04 km² across, the peat is 6 m deep, and the vegetation is dominated by *Sphagnum* moss (*S. angustifolium* and *S. magellanicum*). Vascular plants including ericaceous shrubs (*Chamaedaphne calyculata*, and *Vaccinium corymbosum*), three-way sedge (*Dolichium spp*), and pitcher plants (*Sarracenia purpurea*) are also present in significant numbers. CB is an oligotrophic floating bog of approximately 0.05 km², and is associated with a pond on its east side. Its surface is dominated by *Sphagnum* and *Chamaedaphne calyculata*. The *Sphagnum* species in this bog (e.g. *S. fuscum*, *S. magellanicum*) are responsible for its hummocky surface; and the drier hummocks support ericaceous shrubs (*C. calyculata*). Analysis of vertical peat profiles indicated that CB's earlier stages of peat development were sedge derived and closer to neutral pH for a significant fraction of its existence, while MB's peat development was *Sphagnum*-derived for most of its existence (~9,000 years since the last ice age) and a very short-lived sedge stage (Dettling et al., 2007). Peat samples from shallow layers (circa. 20 cm below surface) were anaerobically collected by triplicate in November 2004 for both sites.

DNA extraction, PCR amplification, cloning, sequencing and phylogenetic analysis

DNA from peat samples were extracted as described previously (Cadillo-Quiroz et al., 2006) with the Power Soil™ DNA kit (MoBio, USA). A fragment from positions 1 of the 16S rRNA to 1100 of 23S rRNA gene was amplified using an E2/*Methanoregula*-specific forward primer: 397-GE2F (5'-CTCCGAGTGCCCGTWAAATC-3', this study) and archaeal specific reverse primer: ArcLSU47R (5'-CTTATCGCAGCTTRSCACG-3') (Bräuer et al., 2006). The components of the PCR mixture were as described previously (Cadillo-Quiroz et al., 2006) with the following PCR conditions: 25 cycles of 94 °C for 40 sec, 50 °C for 1:20 min, and 72°C for 1 min.

Amplification products were examined by electrophoresis on 1% agarose gels for size verification and subsequently cloned with the TOPO TA kit (Invitrogen, USA) as previously described (Cadillo-Quiroz et al., 2006). Two clone libraries per site were constructed for MB and CB with 75 clones in each library. CB and MB sequences containing the 16S rRNA gene plus ITS region were aligned using ClustalX, and trimmed sequence fragments containing only the 16S rRNA gene were aligned with ARB software (Ludwig et al., 2004). Phylogenetic trees were constructed with the neighbor-joining method using the PHYLIP package (Felsenstein, 2002).

Model structure and implementation for simulation of phenotypic and genotypic evolution

An individual based model (IBM) that simulates population growth dynamics was implemented as described by Dieckmann and Doebeli (1999). The model is composed of a logistic population growth equation at the limited capacity of the environment, Gaussian distributed competition between individuals, and random

mutation affecting a phenotypic trait “x”. Each individual, described with a phenotypic trait x , with a given x resource utilization, had a specific carrying capacity ($K_{(x)}$) in its environment. $K_{(x)}$ varied according to a Gaussian function $N(x_0, \sigma_K)$, with the maximum at an intermediate phenotype x_0 and variance σ_K^2 . In polymorphic populations, dissimilar individuals are assumed to interact weakly. Therefore, the model assumed that the strength of competition declines with phenotypic distance according to a Gaussian function $N(0, \sigma_C)$ with a maximum at 0 and variance σ_C^2 . Additionally, the death rate was modeled as dependent on competition and the carrying capacity of the environment. No differences in birth rates were considered. The deterministic dynamics of the resident population of phenotype x are as shown in equations A and B (Fig. 5-1):

$$\begin{aligned} \text{A) } \frac{dN(x, t)}{dt} &= r \cdot N(x, t) \cdot \left[1 - \frac{N(x, t)}{K(x)} \right] \\ \text{B) } \frac{dN(x, t)}{dt} &= r \times N(x, t) \left[1 - \frac{N(x, t)}{K(x)} \int C(x - y) N(y, t) dy \right] \end{aligned}$$

Figure 5-1. Equation for the logistic population growth without incorporating the competition between individuals (A), and incorporating competition among varying individuals within the population (B).

where $N_{(x,t)}$ is the population size at time “t”. The carrying capacity $K_{(x)} = K_0 \cdot \exp(-(x - x_0)^2 / 2\sigma_K^2)$, is the stable equilibrium. When a rare mutant y appears in a resident x at carrying capacity $K_{(x)}$, it competes with the discounted density $C_{(x-y)} \cdot K_{(x)}$, where $C_{(x-y)} = \exp(-(x-y)^2 / 2\sigma_C^2)$ describes the strength of competition between phenotypes.

The equations were implemented as an asexual Individual Based Model (IBM) using the R software (Anonymous). An initial population of 100 identical individuals was assigned an arbitrary value for a phenotypic character x , a birth rate “r” and a

death rate “d” as determined by the following equation: $d = r/K_{(x)} * \sum_y N_{(y,t)} * C_{(x-y)}$, where the sum weights all individuals by their competitive impact on x. Offspring phenotype was implemented as if all mutate within a small range within a normal distribution. Descendants kept their parents' phenotypes except when a mutation occurred (at a rate 0.0001), in which case their phenotype is chosen from a normal distribution $N(x, 0.05)$ where x is the parents' phenotype. Additionally, the DNA sequence of a 250 base fragment from the ITS region of the dominant phylotypes in both sites (CB and MB), was transformed from its A, G, C, and T code into 0 to 3 discrete numbers (each corresponding to a DNA base). The generated numerical sequence was attached to each individual of the initial population within a vector that included a sequence mutation function. Sequences were considered neutral, i.e. with no impact on fitness, and the parental DNA strand inherited had a mutation rate of 0.001 per generation per nucleotide. Mutation among DNA bases was assumed to be uniformly distributed, so the difference in frequency between transition and transversion mutations was not accounted for in the model.

Simulations scenarios and parameters

Simulations were performed for assumed competition scenarios for “E2/*Methanoregula*” populations along the ecological succession changes of the two acidic bogs in this study (MB and CB). The variances of the carrying capacity and competition (σ_K^2 and σ_C^2) among individuals were assumed to change in time as peat conditions, such as pH, mineral content, and decomposition rates among other variables, changed at each site as they developed from pond to fen to bog. Other parameters were kept constant: $r=1$, $K_0=500$, gene sequence mutation rate=0.001. The initial population at each site was 100 individuals with similar phenotype (0.2) and same gene sequences, as is the case when colonizing a new environment. Sequences

were collected at the end of simulations and transformed back into DNA FASTA format for subsequent alignments and phylogenetic tree construction with ClustalX and PHYLIP software (Felsenstein, 2002).

Preliminary short simulations were performed for 500 generations to test initial parameters of ecological and gene diversification. Subsequent simulations were done to 5500 generations.

5.4 RESULTS AND DISCUSSION

E2/*Methanoregula* group microdiversity

Molecular surveys and phylogenetic analyses of environmental 16S rRNA gene sequences from terrestrial and aquatic ecosystems commonly show occurrence of small sequence variations or phylotype “microdiversity”- i.e. highly branching tips on phylogenetic tree (Acinas et al., 2004, Liu et al., 2003, Zhou et al., 2004). Some fraction of this gene microdiversity can originate from PCR-artifacts or multiple rRNA operons with slight variability in a single organism. However, sequence microdiversity is also common among directly-recovered DNA (Cottrell et al., 2005, Liles et al., 2003). In addition, the analysis of sequences of organisms with multiple operon and their sequence variability indicated that in groups with low operon copies the operon variability would not be high enough to account for observed microdiversity patterns (Venter et al., 2004). Moreover, the recently completed genome sequence of "*Methanoregula boonei*" strain 6A* (www.jgi.doe.gov) shows that this organism has a single rRNA operon, so that if this holds true for its close relatives in the bog populations, sequence divergence in multiple operons is not a problem.

The 16S rRNA-ITS gene sequences recovered in this study (Fig. 5-2) showed the occurrence of gene microdiversity patterns within the E2/*Methanoregula* group in acidic bogs.

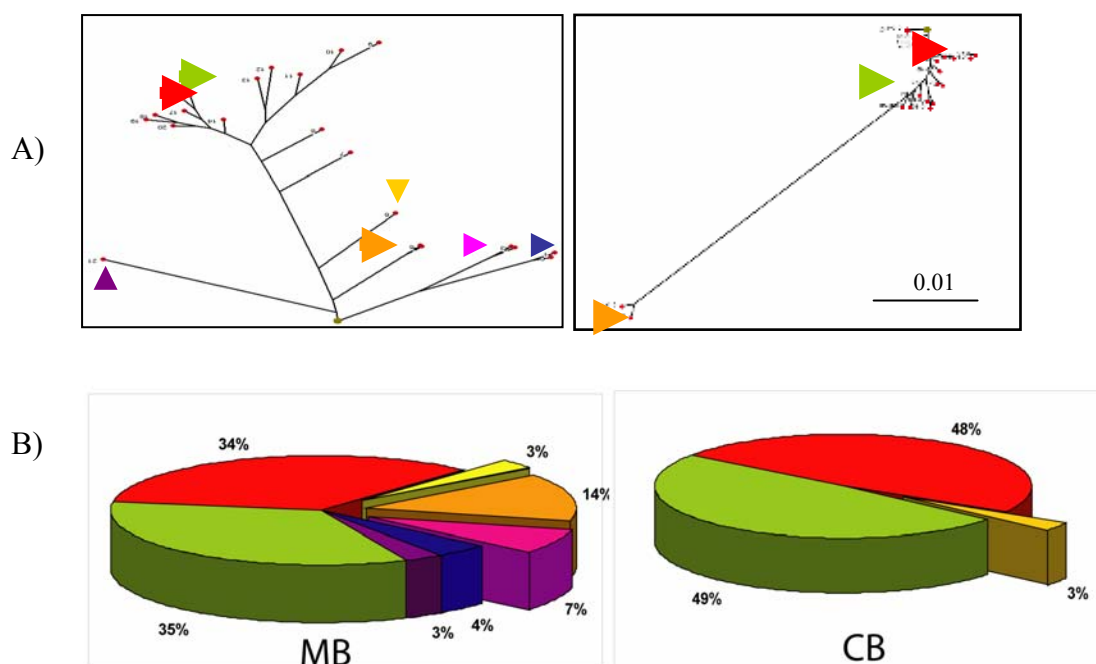


Figure 5-2. 16S rRNA gene phylogenetic tree (A) and clone distribution (B) of “E2/*Methanoregula*” populations in McLean Bog (MB) and Chicago Bog (CB) in November 2004. Red and green fractions are almost phylogenetically identical (circa. 99.6% identity) and are likely members of the same subpopulation. Clone types correspond to positions of colored arrows. Sequences with out an arrow correspond to singletons with potential but unconfirmed chimerical origin.

Fine branching patterns were observed among sequences with approximately 98-99% similarity. Two almost identical sub-cluster (red and green) were highly dominant in both sites and corresponded with another set of sequences called MB-19 from a previous study in Mclean Bog (Basiliko et al., 2003). In that study the MB-03 related sub cluster was suggested dominant over MB-19; however, these results have not been able to confirm by reanalyzing that environmental survey. Previous work in the same sites has shown that E2/*Methanoregula* group is similarly dominant in the two bogs, making up 50 to 90 % of all *Archaea* and reaching population sizes in the order of 10^8 cells/g of dry peat (Cadillo-Quiroz et al., 2006). The large and similar population size of this group in both bogs suggest that the environmental conditions

affecting group fitness (i.e. carrying capacity), in the layers where the samples were obtained, are somewhat similar. Nevertheless, despite similar population size and potentially carrying capacity, the microdiversity patterns of both sites were contrasting: MB had a more diverse subclustering (5-6 subgroups) while CB was less diverse (2-3 subgroups) (Fig. 5-2)

Ecological assumptions and modeling

In an attempt to explain the microdiversity patterns in MB and CB, we implemented an individual based model (IBM) using the adaptive dynamics theory. Long term conditions from the ecological succession in peatlands ecosystems were selected as the source of changing environmental conditions.

Transitions from ponds to fens and fen to bogs, represent a commonly observed ecological succession pattern in peatland ecosystems (Tarnocai & Stolbovoy, 2007). Environmental characteristics in each of these stages are rather different particularly between bogs and the other stages. Ponds (or lakes) and fens commonly present higher pH (near 7) than bogs (4-5), and the vegetation composition and abundance is different in the three stages influencing the type and amount of carbon (Blodau, 2002). The final anaerobic degradation products of organic matter, such as H_2 and CO_2 , are required substrates for methanogenic *E2/Methanoregula*. The physiological characterization of an *E2/Methanoregula* isolate (*Candidatus* *Methanoregula boonei* strain 6A8) demonstrated the potential acidiphilic nature of this group with an optimal pH near 5.0 in culture (Bräuer et al., 2006), and near 4.5 in bog peat incubations (Bräuer et al., 2004). *E2/Methanoregula* is dominant in bogs (Cadillo-Quiroz et al., 2006), but in fens it represents a small fraction of methanoarchaea, as indicated by T-RFLP analysis and clone library analysis (Cadillo-Quiroz et al., 2008), and its presence and proportion in pond or lake stage has not been

specifically addressed, although reports examining *Archaea* at these sites have not reported their presence. Thus, a low E2/*Methanoregula* carrying capacity with high competition among individuals in pond and fen stage, and a higher carrying capacity with lower competition among varying individuals in bog stage, is a reasonable assumption used in the simulations (Fig. 5-5 and 5-6).

The IBM in this study considered the death rate variable and dependent on competition and the carrying capacity of the environment, while the birth rate was similar for all individuals. To test this implementation, two 500-generation simulations were run assuming that all descendants would phenotypically mutate with a genotypic mutation rate of 0.008%. The simulations were done under two scenarios. In the first, the variance of competition was low (individuals compete more within rather than among sub populations) allowing for the segregation of subpopulations into different evolutionary stable phenotypes (Fig. 5-3). By having a broader range of phenotypes, the population increases the range of its fitness landscape and reached higher sizes.

In the second scenario, competition had a higher variance, and individual interference occurred in a broader range limiting ecological diversification (Fig. 5-4). Both simulations showed that genetic diversification was influenced by the change in ecological interactions. When the ecological diversification was broader (Fig. 5-3A), genetic diversity, in addition to being higher (indicated by the numbers of secondary and tertiary branches), also presented more radiating points (star-like branching) (Fig. 5-3B). Secondary branching and radiating points were similar to those in proposed microbial speciation processes (Cohan, 2001). Under Cohan's ecotype proposal, existing genetic diversity undergoes purging processes disrupting speciation (subpopulations are different enough to survive purging conditions and do not strongly compete with the parental group or other species). When competition was broad among variable individuals, this was detrimental to ecological diversification (Fig. 5-

4A) and genetic variation did not form as many star-like clades or secondary branching (Fig. 5-4B).

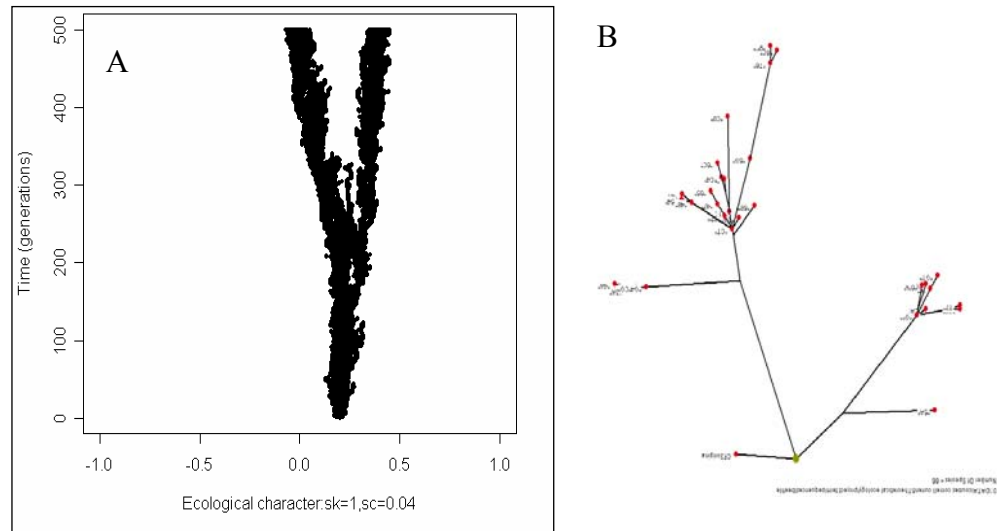


Figure 5-3. IBM simulation with variance of $K_{(x)} = 1$ and variance of $C = 0.04$. Plot of individual with corresponding ecological trait from generation 1 to 500 (A) and phylogenetic tree containing all the sequences of individuals (66) at generation 500 (B).

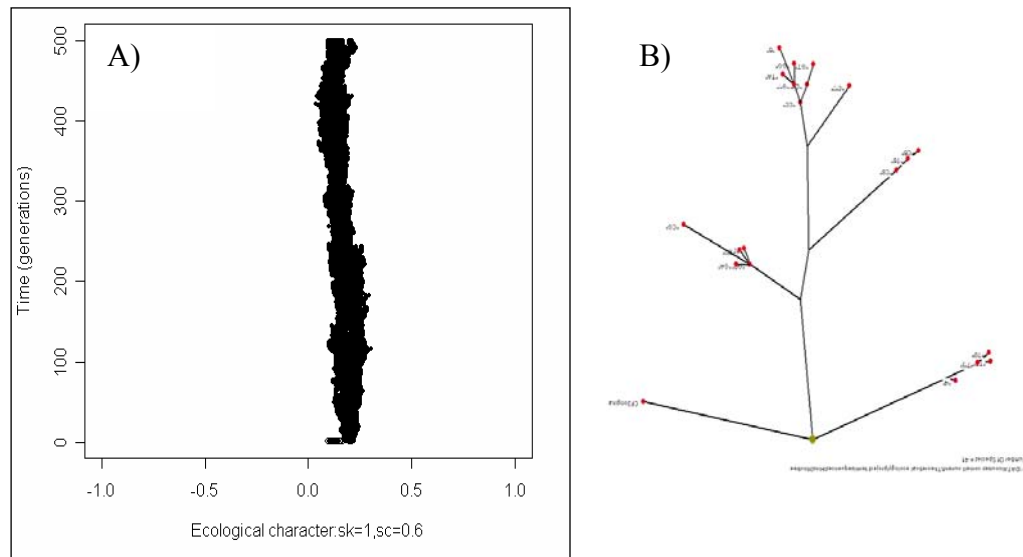


Figure 5-4. IBM simulation results with variance of $K_{(x)} = 1$, and variance of $C = 0.4$. Plot of individual with corresponding ecological trait from generation 1 to 500 (A) and phylogenetic tree containing all the sequences of individuals (50) at generation 500 (B).

The theoretical possibility of ecological succession as the driver of different diversity patterns was explored with a second implementation of the IBM. In the model, not all descendant phenotypes mutate, and those that do so occur at a rate 0.001 and their mutant phenotypes are chosen from a normal distribution from the parents' genotypes. We hypothesized that individuals from E2\ *Methanoregula* were present in CB and MB shortly after the formation of the sites as ponds; and the differences in ecosystem succession stages at both sites limited or allowed diversification by changing the strength of individual-environment and individual-individual interactions in time. These assumptions were based on observations of the low abundance of the group E2 in ponds (personal observation) and fens (Cadillo-Quiroz et al., 2008), which suggest a low and narrow K and likely more spread C since individuals from low density populations likely compete more strongly with different groups than its relatives. The assumed slight differences in the bog stage from both sites attempted to reflect the difference in organic matter decomposition, and therefore substrate flux to methanogenesis, between the two sites. CB is dominated by *Sphagnum* and evergreen shrubs while MB is dominated by *Sphagnum* and a more diverse mix of sedges and shrubs, sedge material has a faster degradation rates in peatlands (Bridgham & Richardson, 2003). The stages were assumed to have different durations in each site (1000, 2500, 2000 generations for MB, and 3000, 1500, 1000 for CB). The duration of each stage was based on a rough extrapolation from analysis of the occurrence of sedimentary, sedge-derived or sphagnum-derived peat. The type of peat and their layers' thickness in the vertical profile are considered to be related to the type and length of each developmental stage. Dating measurements from different peat layers will be performed in the near future to provide a more accurate time frame for the duration of each stage.

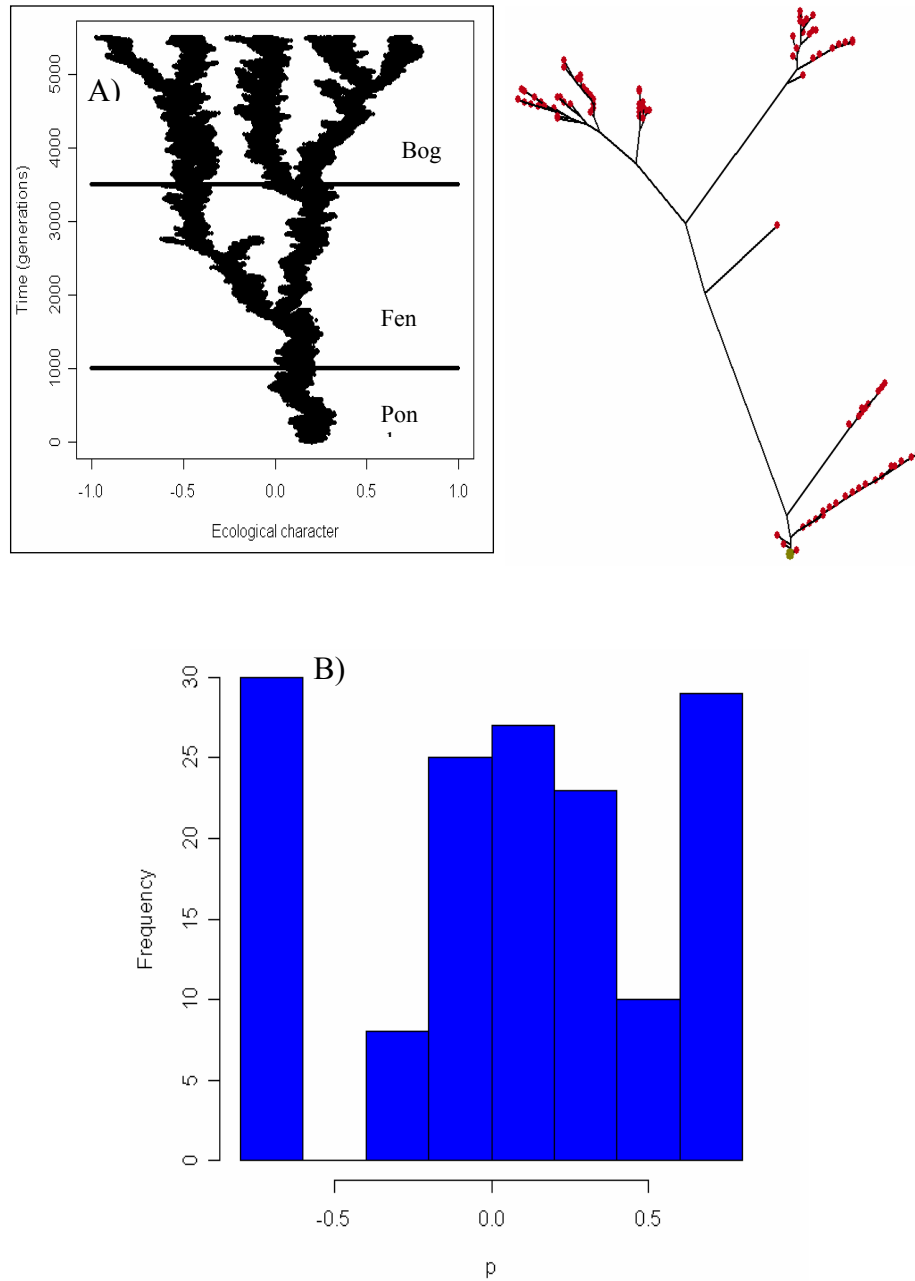


Figure 5-5. IBM simulation for 5500 generations in MB. Parameters were adjusted to reflect ecosystem succession. Parameters at each stage were as follows: Pond→ 1000 years, $\sigma_K^2=1$, $\sigma_C^2=0.6$, initial/fen peatland→1000 years, $\sigma_K^2=1.1$, $\sigma_C^2=0.1$; acidic bog→ 1000 years, $\sigma_K^2=1.3$, $\sigma_C^2=0.04$. A) Ecological phenotype of all individuals at each generation and phylogenetic tree of randomly sampled individuals from last simulation point (115 sequences). B) Histogram of the phenotype (character x) distribution among all surviving individuals (186) at the last simulation point (generation 5500)

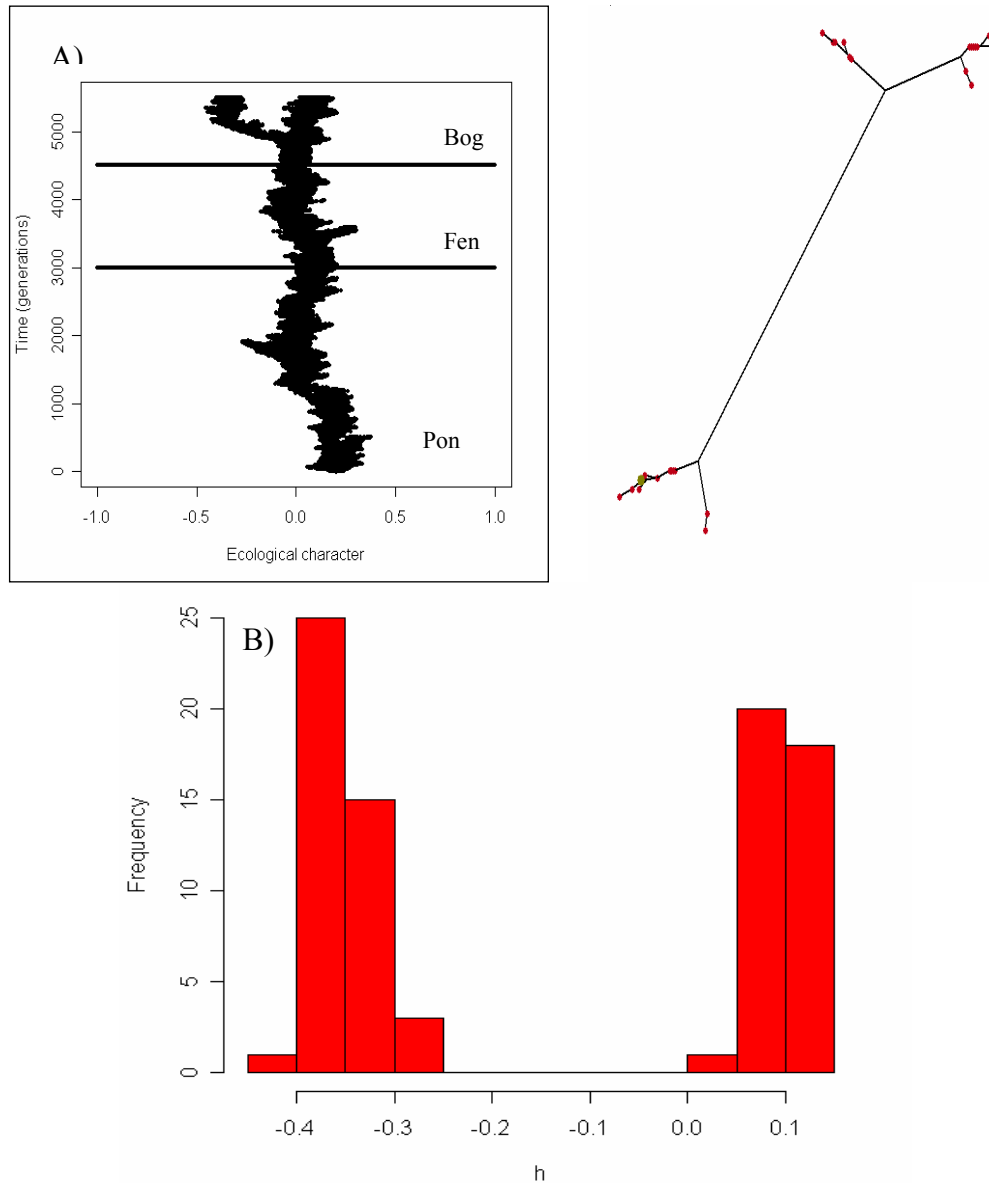


Figure 5-6. IBM simulation for 5500 generations in CB. Parameters were adjusted to reflect ecosystem succession. Parameters at each stage were as follows: Pond→ 3000 years, $\sigma_K^2 = 0.9$, $\sigma_C^2 = 0.4$, fen→1500 years, $\sigma_K^2 = 1$, $\sigma_C^2 = 0.6$; acidic bog→ 1000 years, $\sigma_K^2 = 1.1$, $\sigma_C^2 = 0.09$. A) Ecological phenotype of all individuals at each generation and phylogenetic tree of generation 5500 individuals (99 sequences). B) Histogram of the phenotype (character x) distribution among all surviving individuals (99) at the last simulation point (generation 5500).

The results of MB and CB simulations indicated differences in phenotypic and genotypic diversity in each site (Fig. 5-5 and 5-6). Importantly, simulated gene

sequences had similar patterns of phylogenetic clustering to those observed in environmental sequences from MB and CB (Fig. 5-2A vs 5-5B and 5-6B). In addition, the genotype-to-phenotype relationship was established when analyzing the match between phenotypic distribution of the individuals versus their phylogenetic branching, i.e. groups with similar phylotypes had similar sequences, with only a few exceptions (<1% of total individuals). This suggests that the succession-driven diversity hypothesis is a theoretically possible scenario for the contrasting patterns observed between the two sites.

Although the studies on microbial speciation and maintenance of diversity in natural ecosystems are still scarce, our modeling results are supported by different lines of empirical research. For example, diversity patterns of microorganisms have been suggested to respond primarily to local ecological conditions, such as edaphic factors in soils (Fierer & Jackson, 2006). Microorganisms' fast rates of population growth are considered key for an efficient sorting to ecological conditions. Additionally, microdiverse clusters in marine ecosystems have been proposed to arise by selective sweeps and persist because competitive mechanisms are too weak to purge diversity from within them (Acinas et al., 2004), which is likely to be valid in terrestrial systems as well. Meanwhile, laboratory-based studies using microbial experimental populations have provided evidence that specialization in heterogeneous environments (Rainey & Travisano, 1998) and competition in homogeneous environments (Friesen et al., 2004) lead to diversification. Thus, theoretical scenarios for microdiversity evolution should incorporate factors such as competition and ecological conditions, as in our modeling efforts.

We base our model on the theory of adaptive dynamics (AD) since it effectively incorporates small, individual variability as its basis for adaptive branching (Dieckmann & Doebeli, 1999, Waxman & Gavrillets, 2005). Phenotypic variance has

been recognized as a minimum requirement for evolutionary branching; and it has been shown that depending of the magnitude of the variance, different evolutionary paths can form in the phylogenetic tree (Nishimura & Isoda, 2004). AD is a theoretical approach to studying phenotypic change that take place over time in evolving populations (Waxman & Gavrillets, 2005). The basic assumptions of AD modeling are more likely to be better satisfied by microorganisms than any other group. Microorganisms can more likely satisfy assumption such as (i) clonal reproduction, (ii) small initial mutant frequency in a large resident population and (iii) small mutational steps (Geritz & Gyllenberg, 2005). Using AD's tools, diverse ecological scenarios can be investigated for its ability to give rise to diversification (Doebeli & Dieckmann, 2005). In addition, empirical evidence shows that speciation may arise largely as a byproduct of ecological differences and divergent selection on a small number of phenotypic traits (McKinnon et al., 2004), as considered by AD.

Only one previous report has shown an example of modeling phenotypic branching coupled to genotypic evolution (Semovski et al., 2004). That report demonstrated that using AD coupled to gene sequence simulations, environmental changes can influence the sequences phylogenetic topology and proposed this as a alternative approach to distinguish between scenarios of environmental change that are important for gene diversification or potential speciation processes (Semovski et al., 2004). However, no other report has yet used this type of modeling to address empirical observations as in our study. We certainly expect to continue the development of this type of modeling for the assessment of microbial diversity.

Finally, it is worth mentioning the shortcomings of the simulations in this work for future improvements. Values for K and C variance and their change along time were assumed from very rough estimations and are likely not accurate, so these values

need empirical support in the future. Additionally, although the phylogenetic branching in simulated sequences was consistent with that of environmental sequences, the distribution of their genotypes differed (particularly in CB, Fig. 5-2B and 5B). In the model, after a population diversifies into an ecological stable subpopulation, each group attempts to reach its maximum carrying capacity. This led to observations in the simulations that after a branching split the proportion of individuals becomes $\sim 50/50$, which is not common in real life observations. This apparent limitation of the model leads us to consider the incorporation of different mechanisms of survival for niche-overlapping species and asymmetric competition, among others.

This work represents the first attempt to use an ecological-interactions-based model to assess potential scenarios for the origin of microbial gene microdiversity commonly observed in terrestrial and marine ecosystems.

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CHAPTER VI

COMPOSITION OF EURYARCHAEAL COMMUNITIES IN BOGS AND FENS FROM TEMPERATE AND BOREAL PEATLANDS

6.1. ABSTRACT

CH₄-emitting peatlands are highly concentrated in northern latitudes. Several studies have identified their inhabiting methanogenic *Archaea* in sparsely distributed sites or within a single region. A broader comparison among peatlands from different latitudes has not yet been done. In this study, we assessed the community structure of methanogenic *Archaea*, and related phylogenetic groups, using terminal restriction fragment length polymorphism (T-RFLP) analysis. T-RFLP analysis was highly reproducible among distant samples. In temperate peatlands, we observed that the potentially hydrogenotrophic methanogen group E2/E2' was dominant in particularly acidic bogs (pH 3.8 to 4.4) with the group E1/E1' as a minor community fraction. The proportion of E1/E1' increased as the pH increased in poor fens (pH 4.5-5.5) representing the codominant group with E2/E2'. In near neutral pH minerotrophic peatlands, the E1/E1' group represented a large proportion of the community, as did the potentially acetoclastic methanogens in *Methanosaetaceae* (MT). *Methanosarcinaceae* (MS) was a small fraction in temperate sites. Additionally, several differences were observed in boreal peatlands where MS represented a higher fraction of the community, being the dominant methanogen in some sites, while MT in general represented only a minor fraction. Rice Cluster-II (RC-II) increased in almost all boreal samples, reaching relative values of 20 to 40% far above its values of 1 to 12% from temperate sites. *Methanobacteriales* (MB) also increased its proportion, but particularly so in the northernmost sites.

In summary, this report shows ecological (bog to fen) and geographical (temperate to boreal) patterns in methanogenic *Archaea* community structure in northern peatlands. These patterns should be considered in assessing the potential change of CH₄ emission patterns from peatlands resulting from warming atmospheric conditions.

6.2 INTRODUCTION

Peatlands cover around 5 million km² globally, store near one third of all terrestrial carbon (Gorham, 1991), and contain 10% of global freshwater (Tarnocai & Stolbovoy, 2007). Although peatlands are distributed worldwide (Gore, 1983), their sites are mainly concentrated in northern latitudes along the northern temperate, boreal, sub arctic and arctic regions from North America and Eurasia (Gorham, 1991). Importantly, northern peatlands are estimated to contribute near 20% of the total annual CH₄ emissions (Harriss et al., 1985), and the observed increase of atmospheric CH₄ (Cicerone & Oremland, 1988) contributes to the atmospheric warming registered in the last decades (Hansen et al., 2006).

Methanogenic *Archaea*, are the only organisms capable of CH₄ production (Boone et al., 1993), and they are found inhabiting waterlogged, highly-reduced, and commonly anaerobic environments, such as peat soils and sediments (Garcia et al., 2000). In peatlands, several studies have assessed the methanogenic activity and community composition (Basiliko et al., 2003, Galand et al., 2005, Cadillo-Quiroz et al., 2006, Rooney-Varga et al., 2007, Kotsyurbenko et al., 2007) indicating that occurrence of methanogenesis from acetate or H₂/CO₂, as well as phylotypes associated with known methanogens and novel archaeal clades. These studies have effectively contributed to identifying the methanogens inhabiting peatlands, and some studies have been followed by the isolation of representative strains of novel groups

(Bräuer et al., 2006, Cadillo-Quiroz et al., 2008). However, the assessment of the community composition of methanogenic *Archaea* has been restricted to a few study sites or a narrow geographical region.

In northern latitudes, peatlands are not only distributed in a wide geographical range but also present diverse ecological conditions (Martini et al., 2007). The classification of peatlands into bogs and fens encompasses the main differences among sites. Bogs are commonly oligotrophic, ombrotrophic (rain-fed), acidic, and *Sphagnum*-dominated sites, while fens are minerotrophic, rheotrophic (groundwater-fed), circumneutral or alkaline, and sedge-dominated sites (Gore, 1983). As a result of the varied ecological conditions, differences in the ecological control of methanogenesis have been observed (Blodau, 2002), and different methanogenic community structure has been suggested by molecular surveys (Galand et al., 2003, Galand et al., 2005). Nevertheless, a more detailed comparison of ecologically different peatland is still lacking.

In this study, we assessed the community structure of methanogenic *Archaea*, and closely related groups, from multiple bogs and fens from temperate and boreal latitude sites of North America and Europe. A methanogenic *Archaea*-specific terminal restriction length polymorphism (T-RFLP) we recently developed (Cadillo-Quiroz et al., 2006) was used as a means for community profiling.

6.3 METHODS

Sampling sites and peat collection

The locations of our sampling sites along with several ecological characteristics, such as pH, vegetation, and water table, are described in Table 6-1.

At each site, three samples in ~200 mL airtight canning jars filled to the top were collected along a 30 m transect from the center of the site or from sections with a

large area containing the dominant vegetation type. In peatland complexes such as Lakkasuo, Finland, samples were taken from previously characterized sections with contrasting vegetation and chemistry (Laine et al., 2004). The samples were taken from ca. 15-30 cm depth below surface, targeting the layer that has commonly found with the most active populations of methanogens (Cadillo-Quiroz et al., 2006, Dettling et al., 2007). The depth of sampling was commonly just under the water table, which allowed placing the peat into the jars under submerged conditions, minimizing any potential exposure to oxygen. Samples were transported to the laboratory and processed immediately after arrival. The average time for samples' transportation was 3 to 7 d. Samples were processed inside of an anaerobic glove box (Coy, USA) under a 70%/30% N₂/CO₂ atmosphere.

DNA extraction and PCR amplification

0.5 g duplicate samples from each of the three jars from each site were extracted with the Power Soil™ DNA kit (MoBio, USA) using the manufacturer's protocol including 80 seconds of bead beating at maximum speed (MiniBeadbeater™, Biospect Products, USA). The quality of recovered DNA solution was examined by 2% agarose gel electrophoresis and spectrophotometric readings at 230 and 260 nm; minimal DNA shearing and A_{260/230} ratios close to 1.5- 2.0 were used as criterion for accepting a sample for further analysis.

A fragment from positions 1 to 1100 (*E. coli* numbering) of the 16S rRNA gene was amplified using the archaeal-specific 1AF (5'-TCY GKT TGA TCC YGS CRG AG-3')-1100R (5'-TGG GTC TCG CTC GTT G-3') set of primers (Hales et al., 1996). The PCR mixture contained the following components at its reactants concentrations per µL: 1x Taq buffer with 1.5 mM MgCl₂ (Eppendorf, USA), 0.2 mM deoxy-nucleotide triphosphates (dNTP), 0.25 µM forward and reverse primers, 1.2 U of *Taq* Polymerase

(Eppendorf, USA), 0.2 µg bovine serum albumin (BSA) and 0.1-0.3 ng of extracted DNA. The PCR conditions were as described by Hales et al. (1996) with 25 amplification cycles. Amplification products were examined by electrophoresis on 1% agarose gels for size verification.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

We used the PCR primers and conditions described above. The 1100R reverse primer was fluorescently labeled on its 5' end with Carboxyfluorescein (5'-/6-FAM). T-RFLP analysis was as described previously (Cadillo-Quiroz et al., 2006). Briefly, 30 µL triplicate PCR reactions from each sample were pooled and purified with the Quick Step™2 PCR Purification Kit (Edge Biosystems, USA), and 70 ng were digested with a mix of *HhaI* (15U) and *Sau96I* (10U) enzymes (New England Biolabs, USA) for 3 hours at 37°C. Digested DNA was purified with the Performa® DTRV3 96-Well Short Plate Kit (Edge Biosystems, USA). Purified products were concentrated in a vacuum centrifuge, and then resuspended with a mix of Hi Di-Formamide (Applied Biosystems, USA) and Gene Scan 500-Liz marker (12 µL/mL; Applied Biosystems, USA). Fragments were resolved with an Applied BioSystems 3730xl DNA Analyzer (BioResource Center, Cornell University).

T-RF sequence length, peak height, and area were determined using the GeneScan Analysis Software (Applied Biosystems, 2000) which were exported for profile standardization of each sample. Using a Java-based routine, the relative fluorescent units (RFU) of peaks with lengths of 80 to 400 bases were added by area and normalized to 100 total RFU units. The standardization step minimized peak height and area variation among samples and did not affect the profiles conformation. Standardized profiles of samples from the same site (6 per site) were averaged by their correspondent peak areas, and standard deviations were calculated.

6.4 RESULTS AND DISCUSSION

Peatlands are wetlands where the accumulation of organic matter exceeds its decomposition and where organic matter has accumulated to a layer at least 30-40 cm thick known as peat (Gore, 1983, Glaser, 1987, Blodau, 2002, Martini et al., 2007). This minimum peat accumulation criterion was applied to all sampled sites, and as expected the minerotrophic fens showed less accumulated peat than poor fens or bogs (not shown).

Table 6-1. Sampling sites, location and major characteristics.

Local name, state or country	coordinates	Dominant vegetation	Peat pH	Water table (m)
Baxter bog, Alaska	61° 15' N, 149° 50' W	<i>Sphagnum</i> and shrubs	5.3	0.25
Connors bog, Alaska	61° 11' N, 149° 53' W	Shrubs and some <i>Sphagnum</i>	5.5	0.3
Lakkasuo mire-plot 15 bog, Finland	61° 05' N, 23°50'-24°55' E	<i>Sphagnum</i>	4.5	0.2
Lakkasuo mire-plot 10 fen, Finland		Sedges and <i>Sphagnum</i>	5.6	0.1
Hagesan fen, Norway	ND	Sedges	6	0.3
Stavanger bog, Norway	ND	<i>Sphagnum</i> and shrubs	4.5	0.2
Scheferville, Canada	54° 48' N, 64°49' W	Sedges and <i>Sphagnum</i>	5.9	ND
Dering Lake, Canada	ND	<i>Sphagnum</i> , sedges and shrubs	5.2	ND
Ubberling Mire-bog, Austria	47° 10' N, 13°53' E	<i>Sphagnum</i>	4.5	0.1
Ubberling Mire-poor fen, Austria		<i>Sphagnum</i> , shrubs and sedges	4.7	0.3
Shadow Lake bog, Washington	47° 29' N, 122° 13' W	<i>Sphagnum</i> and shrubs	3.9	0.4
East Edmonds marsh, Washington	47° 05' N, 121° 9' W	Shrubs and <i>Sphagnum</i>	5.5	ND
SF Flambeau Bog, Wisconsin	47° N, 90° W	<i>Sphagnum</i> and shrubs	4.1	0.40
Marsel-2 Bog, Minnesota	47°32' N, 93°28' W	<i>Sphagnum</i> and shrubs	4.2	0.35
Lake Bog, Minnesota		<i>Sphagnum</i>	4.7	0.30
Mer Blue, Canada	45°25' N, 75°31' W	<i>Sphagnum</i> and shrubs	4.3	0.35
North Kemp Fen, Wisconsin	45°50' N, 89°40' W	Sedges	6.6	0.10
Jymie Bog, Wisconsin		<i>Sphagnum</i> and sedges	4.3	0.25
McLean Bog, New York	42°45' N, 76°01' W	<i>Sphagnum</i> and shrubs	4.2	0.15
Chicago Bog, New York	42° 34' N, 76° 14' W	<i>Sphagnum</i> and shrubs	4.2	0.25
Michigan Hollow, New York	42°19' N, 76°29' W	Sedges	7.0	0.05
Big Run Bog, West Virginia	39°07' N, 79°35' W	<i>Sphagnum</i> and sedges	4.2	0.05
Cooper Creek Fen, Georgia	34° 46' N, 83° 57' W	Sedges and some <i>Sphagnum</i>	6.4-6.8	0.04

In our sampling, both bogs and fens were easily located in mid latitudes (39-47°N). However, bogs and minerotrophic fens were scarce in more southern or northern latitudes, respectively. This difference in abundance is determined by broad climatic patterns where the combination of the intensity and length of warm-rainy summers and cold-snowy winters influences the water regime, plant composition, and rate of organic matter degradation, provide favorable conditions for developing either or both type of peatlands (Martini et al., 2007). In terms of geographical range, to our knowledge this study has covered a broader distribution (34°-61°N, 149°W-24°E in Table 6-1) than any singular study assessing the composition of methanogenic *Archaea* in peatlands. Previous studies analyzing *Archaea* (Utsumi et al., 2003, Rooney-Varga et al., 2007) or *Bacteria* (Juottonen et al., 2005, Dedysh et al., 2006, Morales et al., 2006) in peatlands have been restricted to a particular region or sampled only one site per country or continent.

T-RFLP analysis, a PCR-based method, is rapid, highly reproducible, and in the case of simple communities individual fragments can be linked to taxonomic groups (Thies, 2007). Members of the methanogenic *Archaea* are morphologically and physiologically diverse, and form a phylogenetically cohesive group (Garcia et al., 2000); although some nonmethanogenic *Euryarchaeota* are also amplified with the primers we use for T-RFLP. This allowed the development of a T-RFLP set targeting methanogenic archaea, and related groups, with most of the terminal restriction fragment (TRFs) associated to a unique phylogenetic group (Cadillo-Quiroz et al., 2006). Due to DNA extraction and PCR biases, it is important to keep in mind that the *Euryarchaea*-specific T-RFLP used in this study allowed analyses of the community structures of the dominant phylotypes within the scope of the primer sets and by no means represent an exhaustive examination of all euryarchaeal diversity in the sites.

The majority of T-RFs observed in this study were associated with phylogenetic groups previously detected in clone libraries previously reported for several of the sampled sites (Basiliko et al., 2003, Juottonen et al., 2005, Rooney-Varga et al., 2007). TRFs were associated to well-known methanogenic groups such as *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), *Methanospirillaceae* (E2'), and *Methanobacteriales* (MB), or recently isolated methanogenic groups such as Rice Cluster-I (RC-I), the E2/'*Methanoregula*' and E1 groups in the *Methanomicrobiales*, and novel uncultured groups such as Rice Cluster-II (RC-II), Subaqueous Cluster (SC), marine benthic group D (MBD) and group E1'. In addition an average of 2 to 3

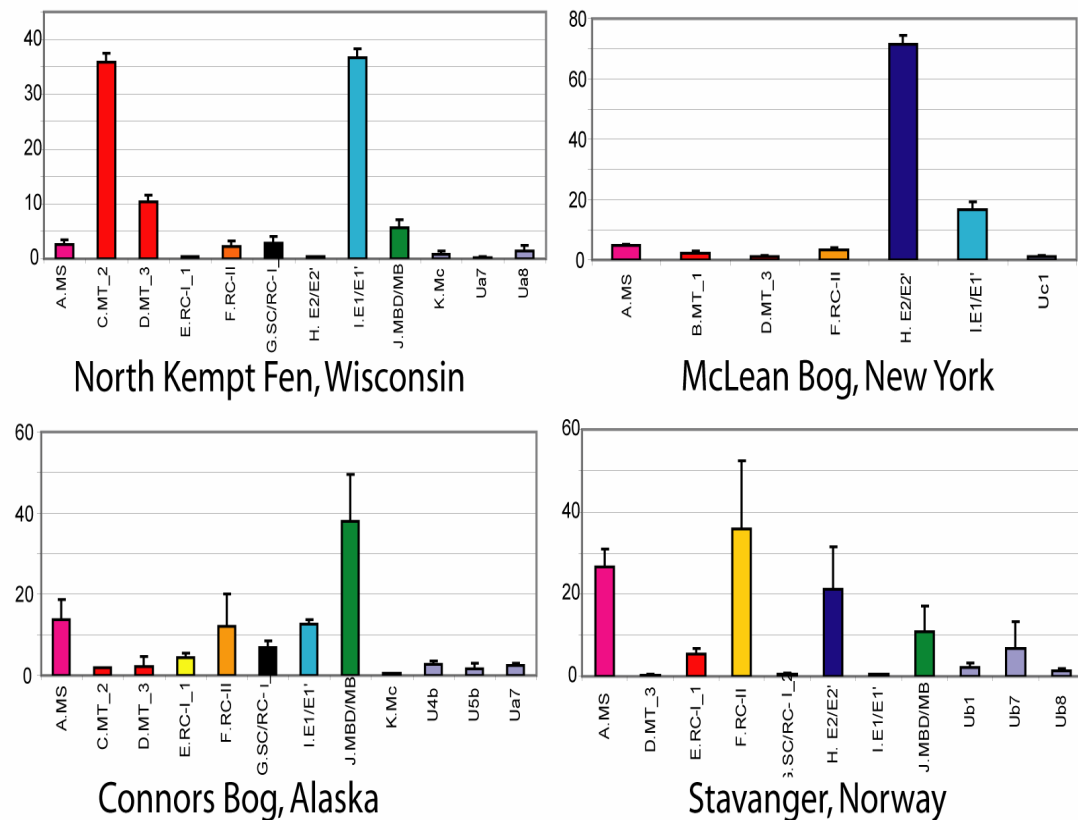


Figure 6-1. Representative T-RFLP profiles after standardization. $N=6 \pm (SD)$. *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), E1 group (E1), group E1' (E1'), group E2 (E2), *Methanospirillaceae* (E2'), Rice Cluster-I (RC-I), Rice Cluster-II (RC-II), Subaqueous Cluster (SC), marine benthic group D (MBD), and *Methanobacteriales* (MB). T-RF assignment was done following a previously described classification (Cadillo-Quiroz et al., 2006)

T-RFs in samples from several sites were not associated with any sequences in our database and usually represented a small fraction (<2% of total relative fluorescence units) (Fig. 6-1). These unidentified TRFs could represent novel clusters within the observed groups or novel groups (both with low abundance) or may have an artifactual source.

As shown by the low standard deviation values in Figure 6-1, T-RFLP traces were highly reproducible among samples located several meters apart (up to 30-50 m). In addition, we observed a contrasting composition among selected profiles from a temperate acidic bog (Chicago Bog), and neutral minerotrophic fen (North Kempt), and boreal poor fens (Stavangen Site and Connor's Site) (Fig. 6-2). When analyzing all the samples by their type and corresponding locations several patterns are apparent for the methanogenic community structure in northern peatlands (Fig. 6-2).

In temperate circumneutral pH (6-7) sedge-dominated sites (4, 12, 14, 18 in Fig. 6-2), potentially acetate-utilizing methanogenic groups, MS and particularly MT, together with the potentially H_2/CO_2 utilizing methanogen group E1/E1' represented the dominant fraction of methanogens. While in the most acidic (pH 3.8-4.5) *Sphagnum*-dominated sites (5, 9, 10, 11, 16, and 17 in Fig. 6-2) the potentially H_2/CO_2 utilizing methanogen E2/E2' group was dominant. In these sites the E1/E1' group often found as a minor or close fraction to E2/E2'. In some cases where the bog pH was near the upper pH value of 4.5, the E1/E1' group was observed in higher proportions than E2/E2' (6, 13 and 17 in Fig. 6-2) although together both groups still represented the dominant fraction. These patterns are consistent with several studies suggesting that methanogenesis from H_2/CO_2 was more important in acidic bogs while acetate utilizing methanogenesis was more important in substrate-rich minerotrophic sites (Hornibrook et al., 1997, Chasar et al., 2000, Martens et al., 2003, Galand et al.,

2005, Vasander & Kettunen, 2006). Phylotypes related with the E2 group also called “Fen Cluster” have been found highly abundant on acidic bogs (Basiliko et al., 2003, Galand et al., 2005, Juottonen et al., 2005) while MT has been seen as dominant over MS on a sedge-dominated minerotrophic fen (Cadillo-Quiroz et al., 2008). The E1 group made up a significant fraction in minerotrophic fens and less acidic bogs or poor fens (pH near 7 and near 4.5 respectively), suggesting that this group is of importance for fens and bogs and sites with intermediate conditions. A recent isolate associated to the group E1, tentatively named *Methanosphaerula palustris* E1-9c, grew on H₂/CO₂ from pH <4.8 to 6.4 with an optimum pH of 5.5-5.7 (Cadillo-Quiroz et al., 2008). The dominance of E2/E2', and minor presence of E1/E1' in bogs with the lowest pH is supported by the H₂/CO₂ utilizing methanogenic growth of isolate '*Candidatus* Methanoregula boonei', a member of the E2 group, with pH growth range from 4.0 to 5.5 and optimum near 4.8 (Bräuer et al., 2006).

The sampled sites in boreal latitudes were commonly poor fens with pH values in the range of 4.5 to 5.6, and mixed vegetation dominated by *Sphagnum* and shrubs (Table 6-1). Although boreal sites showed similar types of T-RFs than lower-latitude peatlands, the methanogenic community structure had marked differences with their temperate counterparts (Fig. 6-2). The proportion of MS increased to major fractions at several sites (2, 3, 8, and 19 in Fig. 6-2) while MT generally represented a small fraction. Interestingly, the increase of the RC-II fraction was common at most of the high-latitude sites (2, 3, 7, 8, 15, 21, and 21 in Fig. 6-2), reaching values near 20 to 40% of the community T-RFs, much higher than the 1 to 12% observed in temperate sites. The MB group also showed an increase in its proportion with a maximum value in the northernmost Alaskan sites (19 and 20 in Fig. 6-2). The E1/E1' and E2/E2' groups represented a smaller proportion of the archaeal community at high latitude sites, particularly those from North America.

The increase in the MS over MT fraction at boreal sites is supported by the accumulation of acetate suggested in high-latitude peatlands (Duddleston et al., 2002) which correlates with the higher acetate utilization thresholds in MS compared to those of MT (Jetten et al., 1992). However, in contrast to our results showing MS as an important fraction of methanogenic *Archaea* in boreal peatlands, another study using a different profiling technique (denaturing gradient gel electrophoresis, DGGE) did not detect MS in 15 Alaskan peatlands with varying vegetation and pH (Rooney-Varga et al., 2007). Several alternatives could explain these differences: (1) phylogenetically different sequences can migrate or comigrate to similar band positions and selective band cutting and cloning of selected samples can overlook the occurrence of some groups therefore MS could have been overlooked in the other study, (2) the addition of GC clamp could have biased the primer against MS and the group was overlooked, (3) MS dynamics made it below the limit of detection of DGGE at their sampling time, and (4) the group is not a significant fraction in Alaskan peatlands and our T-RF matching failed to do a correct identity pairing. To test the last option a clone library could be built for representative Alaskan samples seeking for MS phylotypes and evaluating their predicted T-RF matching.

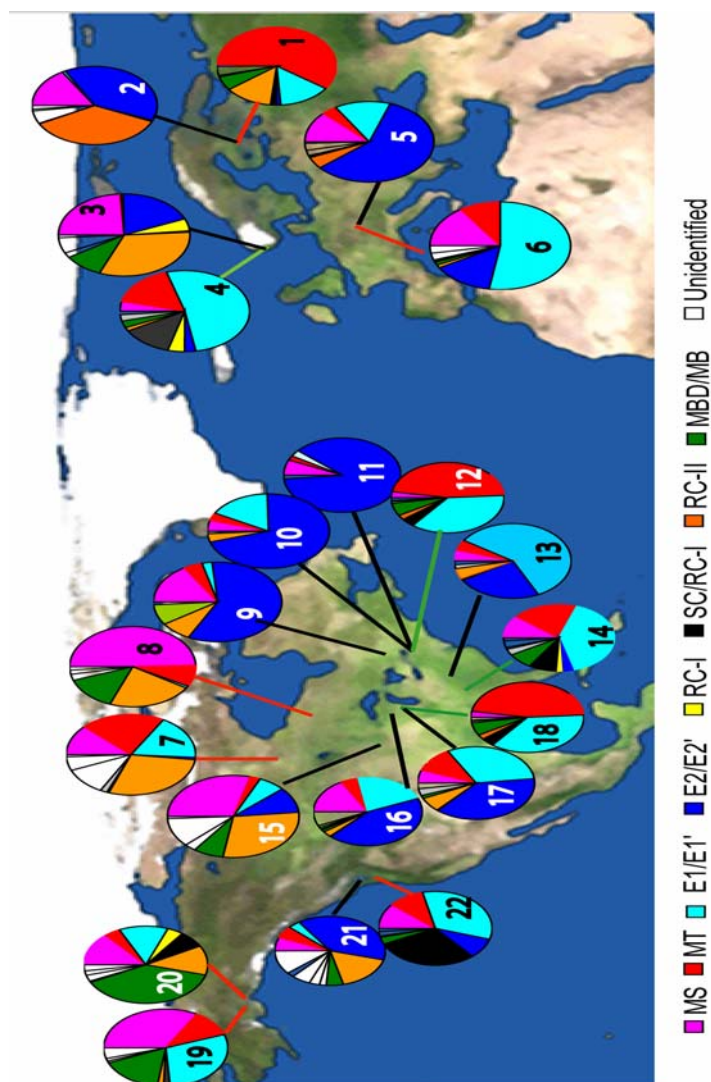


Figure 6-2. Summarized community structure of methanogenic *Archaea* from peatlands in North America and Europe. *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), E1 group (E1), group E1' (E1'), group E2 (E2), *Methanospirillaceae* (E2'), Rice Cluster-I (RC-I), Rice Cluster-II (RC-II), Subaqueous Cluster (SC), marine benthic group D (MBD), and *Methanobacteriales* (MB). 1: Lakkasuo mire-plot 10 fen, Finland, 2: Lakkasuo mire-plot 15 bog, Finland, 3: Stavanger bog, Norway, 4: Hagesan fen, Norway, 5: Ubberling Mire-bog, Austria, 6: Ubberling Mire-poor fen, Austria, 7: Scheferville fen, Canada, 8: Dering Lake, Canada, 9: Mer Blue, Canada, 10: McLean Bog, NY, 11: Chicago Bog, NY, 12: Michigan Hollow, NY, 13: Big Run Bog, WV, 14: Cooper Creek Fen, GA, 15: Marsel-MS2 Bog, MN, 16: SF Flambeau Bog, WI, 17: Jymie Bog, WI, 18: North Kemp Fen, WI, 19: Baxter bog, AK, 20: Connors fen, AK, 21: Shadow Lake bog, WA, 22: East Edmonds marsh, WA. Colored connecting lines symbolize the type of peatland site: circumneutral minerotrophic fen (green), intermediate poor fen (red) and acidic bog (black)

RC-II has been previously detected in temperate (Cadillo-Quiroz et al., 2006) and some boreal peatlands (Kotsyurbenko et al., 2004) but no study until now has pointed to a preference or specialization for a particular type of ecosystem. RC-II was initially detected inhabiting rice roots (Großkopf et al., 1998), however no isolate or enrichment has been obtained for this group which deters us from further explanations about their abundance. Nevertheless, the ecological properties of boreal peatlands should be considered for attempting to isolate this group. Finally, members of the *Methanobacteriales* have been isolated from varied terrestrial and marine sources (Boone et al., 2001), and isolates have been obtained from a temperate and a boreal site (Zellner et al., 1988, Kotsyurbenko et al., 2007). *Methanobacterium* sp. MB4, isolated from the boreal, site has been found to grow under acidic conditions (pH range 3.8 to 6.0, and optimum 5.0-5.5) from bogs and poor fens. *In vitro* experiments has suggested a potential role under acid conditions (Kotsyurbenko et al., 2007), but its *in situ* contribution has not been evaluated.

Although recent calculations suggest that peatlands' CO₂ sequestration and CH₄ emissions nearly balance each other out in their “global warming potential” (Roulet, 2000), the ongoing temperature increase in northern latitudes is expected to change this balance (Hansen et al., 2006). Our results indicate the occurrence of different patterns of archaeal community structure between bogs and minerotrophic fens in temperate sites, and temperate versus boreal sites. These different community structures will likely influence the contribution of the different methanogenic pathways, and should be considered when evaluating the potential responses of methanogenesis to changing environmental and biological factors associated to the atmospheric warming.

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APPENDIX A

**MOLECULAR DIVERSITY OF ARCHAEA INHABITING THE
RHIZOPLANE OF TWO VASCULAR PLANTS FROM AN ACIDIC BOG**

A.1 ABSTRACT

Several studies have observed that the presence of vascular plants can affect CH₄ dynamics in ecosystems such as rice paddies or peatlands. However, the root-associated archaeal communities in CH₄ producing peatlands have not been assessed. In this report, we examined the diversity and structure of the archaeal community inhabiting the rhizoplane of two contrasting vascular plants from an acidic bog in upstate NY: *Dulichium* spp. ('three-way sedge') and *Sarracenia purpurea* ('pitcher plant'). 16S rRNA gene libraries, generated with multiple primer sets, showed the presence of a diverse archaeal community with groups similar to those previously observed in acidic bogs. However, the rhizoplane community had a very different structure from that of the peat soil. The rhizoplane's archaeal community was evenly distributed for both plants between phylotypes associated to Rice cluster (RC) -I, group E2/'*Methanoregula*' (E2) and *Methanosarcinaceae* (MS). Interestingly, a subcluster of RC-III was abundant in *Dulichium* but minimally present in *Sarracenia*, while a MS subcluster was dominant in *Sarracenia* but not *Dulichium*. Group E1, although as a minor fraction of the community, was more abundant in *Sarracenia*. *Methanosaetaceae*, *Methanobacteriales*, and crenarchaeal groups 1.1c and 1.3b were minor components in the rhizoplane clone libraries of both plants. Complementary, PCA analysis of T-RFLP traces showed differential clustering related to both type of host plant and to vertical distribution along the root. These results suggest the rhizoplane of vascular plants as a differentiated and uncharacterized niche for methanogens inhabiting acidic bogs.

A.2 INTRODUCTION

Bogs are *Sphagnum*-dominated peatlands with oligotrophic, ombrotrophic (rain fed), and acidic conditions (Crum, 1992). These ecosystems are abundant in northern latitudes (Martini et al., 2007), store up to a third of all terrestrial carbon, (Gorham & Jannsens, 1992), and contribute 5-10% of the total CH₄ emissions to the atmosphere (Wahlen, 1993). CH₄ is an important greenhouse gas contributing to the atmospheric warming observed in recent decades (Hansen et al., 2006), and *Archaea* is the only group capable of biological production of CH₄. Understanding the ecology of *Archaea* in CH₄-producing ecosystems is necessary for predicting future CH₄ emissions.

Since CH₄ production is a strictly anaerobic process, the root surfaces and adjacent soil have not been considered likely compartments for active methanogenic populations. However, several reports from rice paddies showed that CH₄ is formed in this compartment (Lu et al., 2005) and that it harbors several novel uncultured archaeal groups, including Rice Cluster groups I-VI (Grobkopf et al., 1998a). Additionally, the high rate of oxygen consumption by heterotrophic microorganisms near root surfaces (van Bodegom et al., 2001) and the active transfer of plant carbon to the microbial communities inhabiting roots (Mitchell et al., 2003) provides suitable conditions for the flow of carbon into CH₄ production. Thus, root surfaces can be important for CH₄ production in plants growing under flooded conditions.

Some studies on peatlands have found that the presence of vascular plants significantly affected the release of CH₄ (Kelker & Chanton, 1997), indicating a positive correlation between the presence of sedges, such as *Eriophorum vaginatum*, and higher CH₄ emissions (Greenup et al., 2000). These observations are not surprising, since vascular plants can enhance CH₄ production in their rhizosphere

through the release of exudates (Saarnio et al., 2004). Several other studies have been directed at identifying the methanogens inhabiting bulk peat soil from acidic bogs and novel groups have been identified (Basiliko et al., 2003, Galand et al., 2003, Kotsyurbenko et al., 2004), and later brought to culture (Bräuer et al., 2006). However, despite the potential importance of root surfaces of vascular plants in CH₄ dynamics, no study has assessed the archaeal community inhabiting the rhizoplane of vascular plants from acidic peatlands.

The goal of this study was to characterize the diversity and composition of the archaeal communities inhabiting the root surface of two dominant but physiologically contrasting vascular plants from an acidic bog in upstate NY (McLean Bog). *Dulichium spp.* ('three-way sedge') and *Sarracenia purpurea* ('pitcher plant') were selected for this study.

A.3 MATERIAL AND METHODS

Study site and sampling

The study site, McLean Bog (MB), is located in Tompkins County, NY (42° 05'N, 75° 00'W) and belongs to a *Sphagnum*-dominated chain of peat lands spread along a latitudinal gradient in North America (Fisk et al., 2003). MB is a small (70 m diameter) kettle hole type ombrotrophic bog, with acidic pH (3.6-4) and near 8 m of accumulated peat as described by Osvald 1970. The vegetation in MB is dominated by *Sphagnum* mosses accompanied by vascular plants (Osvald, 1970). Evergreen shrubs (*Ericaceae* family), pitcher plant (*Sarracenia purpurea*), and three-way sedge (*Dulichium spp.*) are the main vascular plants at MB. Cotton grass (*Eriophorum vaginatum*) was also present in MB but with a sparse distribution. Sedge density has increased in recent years compared to previous observations.

Dulichium spp. and *Sarracenia purpurea* were selected for this study because of their abundance, different morphology, physiology, and accessibility. Samples were taken in October 2003, and the vegetation showed signs of fall senescence. Three sampling points containing only one type of plant in a radius of at least 1 m were selected for sampling. The average distance between close sampling points was ~12 m. Samples were retrieved by cutting a block of peat containing the complete root system of a selected adult plant (15-20 cm deep) with ca. 5 cm of surrounding peat (10 x 10 x 20 cm blocks). Two plants of each type were taken at each sampling point. The peat blocks were deposited in sterile polypropylene bags, transported to the laboratory at 4°C and immediately processed for peat separation from roots. The peat surrounding the roots was manually removed, after which the roots were gently rinsed with sterile water.

Rhizoplane sample standardization, cell separation and DNA extraction

In order to standardize the rhizoplane sample size, root surface was measured for several samples of each plant using a high resolution scanner and image analysis with the MacRHIZO software as previously described (Himmelbauer et al., 2004, Zobel, 2003). The linear correlation between root length and root surface was established for each type of root ($r = 0.99$, $p < 0.01$), and an estimated area of 25 cm² root surface was used for cell separation and DNA extraction (Fig. A-1).

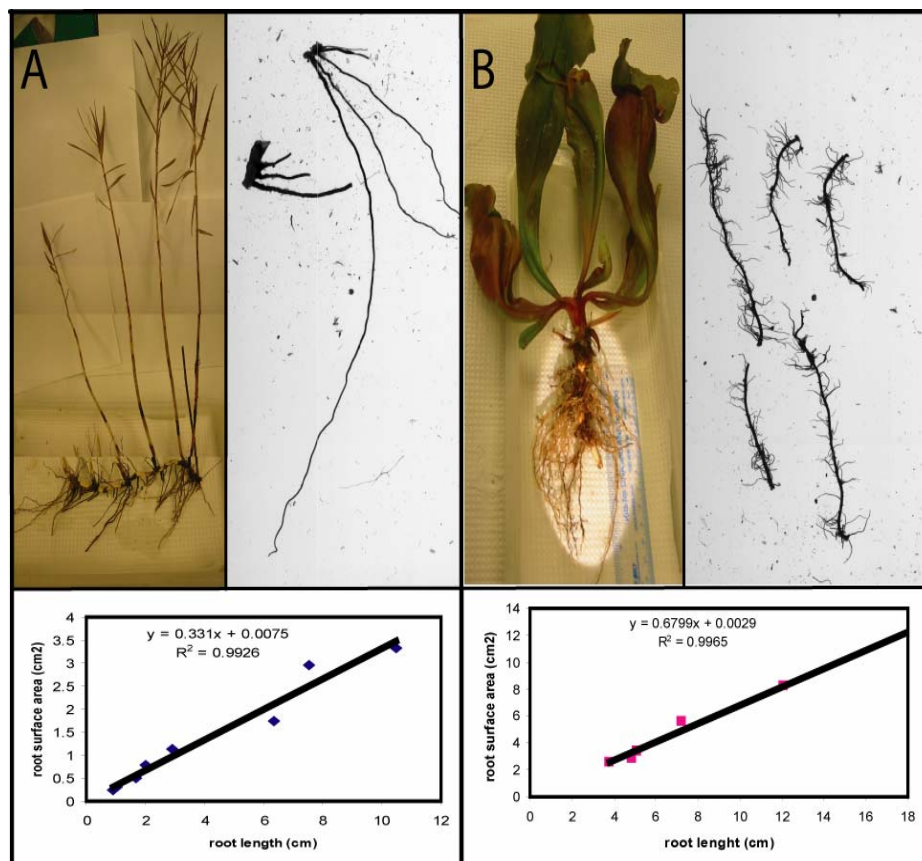


Figure A-1. *Dulichium* spp. (three-way sedge) (A) rhizome-stolon-based root system and *Sarracenia purpurea* (pitcher plant) (B) vertical root morphology and their linear correlation between root length and root surface area.

To avoid co-extraction of plant DNA and subsequent interference in PCR reactions (not shown), the microbial cells associated with the root surface were released into a liquid phase by gentle sonication and bead-vortexing. Roots (25 cm²) were placed in 10 mL tubes containing 6 mL of sodium pyrophosphate solution (0.01%) and 5 glass beads (0.5 cm). The tubes were subjected to 5 cycles of 1 min sonication (Branson 510 Bath Sonicator at 135 W) and 1 min vortexing. The remaining roots were disposed, and the liquid phase was concentrated by centrifugation (20 min at 14 000 rpm.). Pelleted material was used for DNA extraction.

DNA was extracted with the Soil Clean DNA® extraction kit (Qbiogene) following the manufacturer's instructions. DNA was quantified by image analysis of

DNA stained with 0.01 % ethidium bromide using the Fluor-S imaging system and accompanying Quantity One® software (BIORAD).

16S rRNA amplification, cloning and phylogenetic analysis

Three archaeal 16S rRNA gene primer sets (Table A-1) were used in PCR reactions with the following conditions: 5 min at 94°C, followed by 26 cycles of 1 min at 94°C, 1.5 min at correspondent annealing temperature (Table A-1) and 72°C for 1.5 min, plus a final step of 6 min at 72°C. PCR reactions (50 µL) contained the following reactants: 1x Taq Buffer (Eppendorf), 1.5 mM MgCl₂, 50 µM of each dNTP, 0.2 µM of each primer, 1.2 U of *Taq* Polymerase (Eppendorf), 0.2 µg bovine serum albumin (BSA) and 0.2 ng of soil DNA. PCR products were verified by 1% agarose gel electrophoresis.

Table A-1. 16S rRNA gene primers used in this study. A.T.: Annealing temperature.

Name	Target position ^a	Estimated coverage	A.T. (°C)	Sequence (5' → 3')	Reference
A751F	751-768	<i>Archaea</i>	53	GCTAAAGSRICCGTAGC	Baker et al., 2003
UA1204R	1204-1221		53	TTMGGGGCATRCIKACCT	
IAF	1-19	Mainly <i>Euryarchaeota</i>	55	TCYGKTTGATCCYGSCRGAG	Hales et al., 1996
1100R	1100-1115		55	TGGGTCTCGCTCGTTG	
89Fb	89-103	Mainly <i>Crenarchaeota</i> and some <i>Euryarchaeota</i>	48	ACGGCTCAGTAACRC	Buckley et al., 1998
Arc915R	915-934		48	GTGCTCCCCGCAATTCCT	

Clone libraries were constructed for each plant rhizoplane with the three different primer sets (6 libraries). Using the TA Cloning kit® (Invitrogen, USA) and m13 primer screening, 40 clones per library were selected for restriction analysis with *Hae*III and *Hha*I enzymes (New England Biolabs, USA). Clones displaying unique restriction patterns were sequenced with an ABI 3730 automated sequencer (Bio Resources Center, Cornell University). Sequences were compared with the GenBank

database (Benson et al., 2004) in order to ensure that newly-reported related sequences were included in our analysis.

Sequence phylogenetic analyses were done using the ARB software (Ludwig et al., 2004) and a manually updated sequence database. Phylogenetic placement was done using the quartet PUZZLE method implemented in ARB with an 'archaeal' nucleotide base frequency filter that included positions with more than 50% invariance. Tree topology was confirmed using maximum likelihood and neighbor joining methods as implemented in the ARB package.

Terminal-restriction fragment length polymorphism (T-RFLP) analyses

For T-RFLP analyses, *Sarracenia* root samples were subdivided into three categories according to vertical position with reference to the beginning of the stem section of the plant. Roots were divided into upper (0-5 cm), middle (5-10 cm), and deep (11-25 cm) sections, and processed as described above.

The 1AF-1100R primers were labeled on their 5' end with Carboxyfluorescein (5'-/6-FAM) and PET (5'-PET® Applied Biosystems), respectively. PCR conditions were used as described above. T-RFLP analysis was done as follows for each sample: PCR products of three reactions were pooled and purified with the QuickStep™2 Purification Kit (Edge Biosystems, MD). 70 ng of purified product were digested with *Sau*96I restriction enzyme for 5 h at 37°C (New England Biolabs). Digested PCR products were subjected to an additional cleanup step using the Performa® DTR V3 96-Well Short Plate Kit (Edge Biosystems, MD). Purified reaction products were concentrated by vacuum centrifugation (40 min at 5000 rpm), resuspended in a mix of Hi Di-Formamide (Applied Biosystems, USA) and Gene Scan 500-Liz marker (12 µL mL⁻¹; Applied Biosystems, USA). Fragments were resolved with an Applied BioSystems 3730xl DNA Analyzer (BRC, Cornell University). T-RF sequence length,

peak height, and area were determined using the GeneScan Analysis Software (Applied Biosystems, 2000).

Statistical analyses

T-RFLP traces from all samples were compared in an Excel spreadsheet matrix using a ± 1 base peak size binning function. The presence or absence of each peak in each sample was registered as a 0 or 1 in a matrix format. The presence/absence matrix generated was exported and subsequently analyzed with principal component analysis (PCA) using the Canoco® software (Microcomputer Power, NY) as described previously (Devare et al., 2004).

A.4 RESULTS

Archaeal community composition in rhizoplane

16S rRNA gene libraries revealed a diverse archaeal community associated with the rhizoplane of *Dulichium spp* ('three-way sedge') and *Sarracenia purpurea* ('pitcher plant'). The majority of phylotypes were related to euryarchaeal groups (Fig. A-2), although *Crenarchaea* were also detected (Fig. A-3). Phylotypes were associated with well known methanogen groups such as *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), *Methanobacteriaceae* (MB), and recently cultured methanogens such as group 'E2/*Methanoregula*' (E2) (Bräuer et al., 2006), group 'E1' (Cadillo-Quiroz et al., 2008) and Rice Cluster-I (RC-I) (Sakai et al., 2007) (Fig. A-2). Phylotypes related with uncultured euryarchaeal, RC-III, and crenarchaeal, 1.3b and 1.1c, groups (Jurgens et al., 2000) were also detected (Fig. A-3).

Clone distribution analysis showed that in the rhizoplane archaeal community of both plants MS, RC-I and E2 contributed nearly 70-78%, with similar values in the contribution of RC-I (21-20%) and E2 (20-24%) (Fig. A-4). MS represented a larger fraction of the *Sarracenia* rhizoplane community due to the presence of a second MS

subcluster (14%, MS-2 in Fig. A-2 and A-4), when using a 97% sequence identity criterion. MS-2 was present in *Dulichium* only as a very small fraction (3%), and MS-1 was present in both plants at similar proportions (21-24%). RC-I was similarly divided in two subclusters (1 and 2 in Fig. A-2 and A-4) and both had similar values in libraries from the two plants. In contrast, the RC-III cluster had a differential contribution to each plant's rhizoplane community, especially subcluster 1 (Fig. A-4) which represented 23% of the clones in *Dulichium* libraries and only 1% in *Sarracenia*. The contribution of the group E1 was slightly higher in *Sarracenia* libraries (8%) than in those from *Dulichium* (3%). MB was only detected in *Sarracenia* as a 5% fraction. The detected crenarchaeal phylotypes represented a small fraction of rhizoplane community from *Dulichium* and *Sarracenia*. The group 1.3b (Jurgens et al., 2000) was a 3% fraction in both plants, while group 1.1c was only present in *Sarracenia* as 3% (Fig. A-4).

Analysis of diversity coverage by different primers

None of the primer sets used in this study recovered phylotypes from all archaeal groups (Fig. A-5). The 1AF-1100R primers recovered only euryarchaeal sequences from almost all groups excepting MS-1 and RC-III (Fig. A-1 and A-2). Meanwhile, 89Fb-915R had the broadest coverage, retrieving euryarchaeal as well as crenarchaeal sequences and only missing the groups MT and RC-III _1. Primers A571-1204 recovered euryarchaeal and crenarchaeal groups as well, but they missed several groups such as MS-2, MT, RC-I_1, RC-III_2, MB and G1.1c.

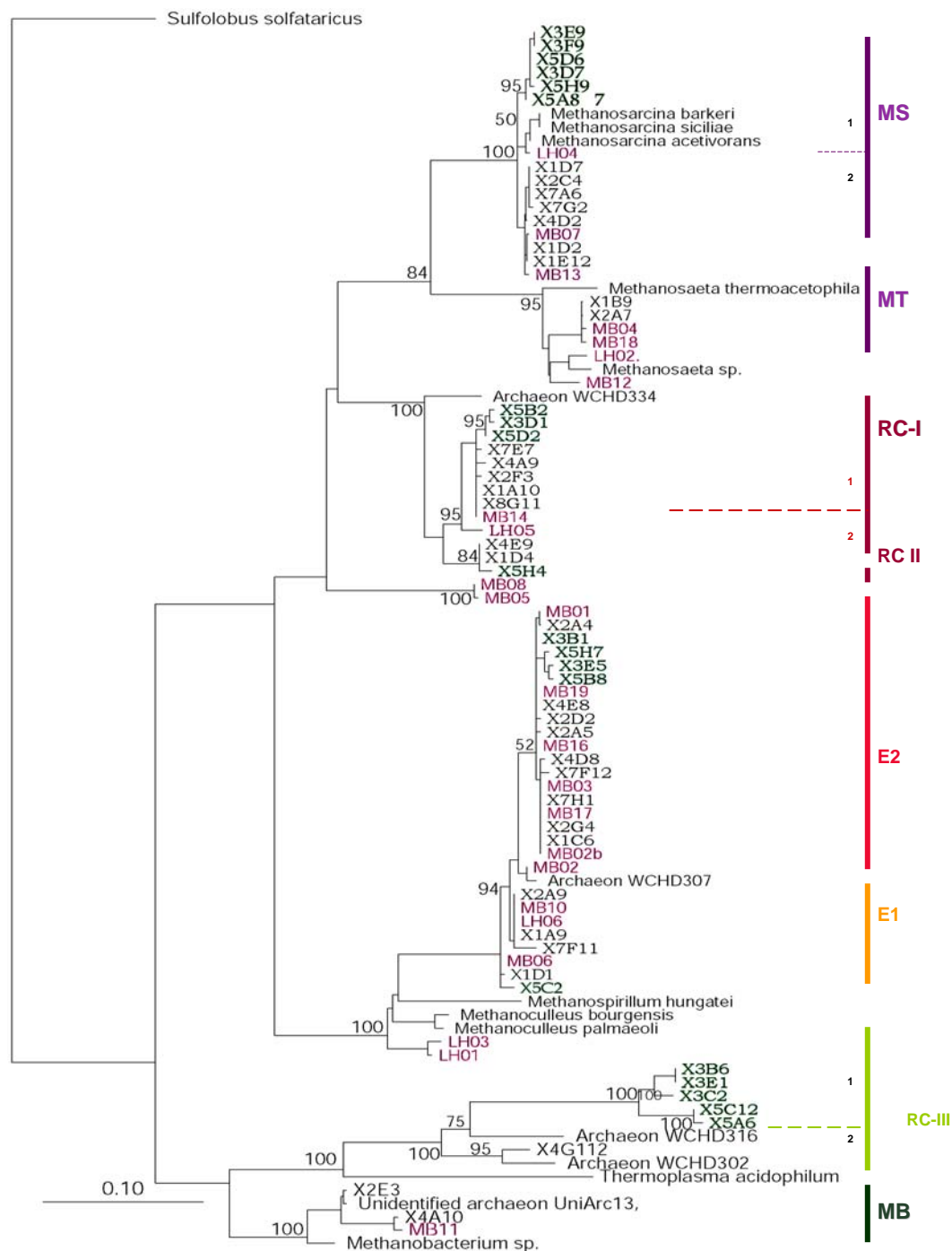


Figure A-2. Phylogenetic tree of euryarchaeal 16S rRNA gene sequences. MS: *Methanosarcinaceae*, MT: *Methanosaetaceae*, RC-I: Rice Cluster I, RC-II: Rice Cluster II, E2: group E2/Methanoregula, E1: group E1, RC-III: Rice Cluster III, and MB: *Methanobacteriales*. 1 and 2 represent sub clustering at 97% sequence similarity within a group. All rhizoplane clone names start with x, and their colors indicate their source: green for *Dulichium* and black for *Sarracenia*. Names colored magenta corresponded to clones sequences from bulk peat soil by a previous study in MB (Basiliko et al., 2003).

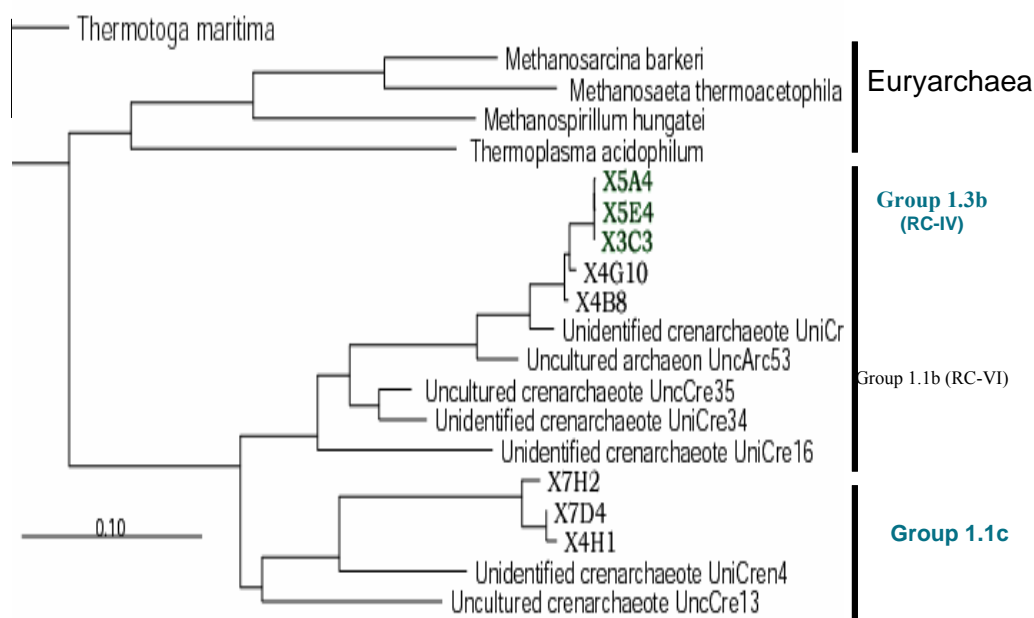


Figure A-3. Phylogenetic tree of crenarchaeal 16S rRNA gene sequences. All rhizoplane clone names start with X and their name colors indicate their source: green for *Dulichium* and black for *Sarracenia*.

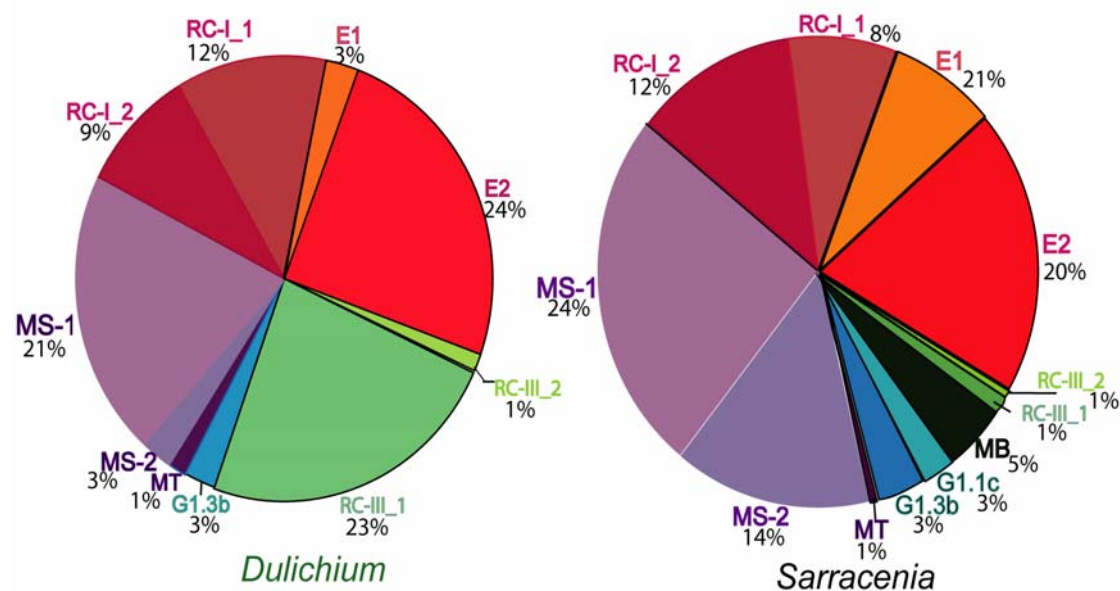


Figure A-4. Clone distribution of combined 16S rRNA gene libraries from *Dulichium* and *Sarracenia* rhizoplane samples. MS: *Methanosarcinaceae*, MT: *Methanosaetaceae*, RC-I: Rice Cluster I, RC-II: Rice Cluster II, E2: group E2/'Methanoregula', E1: group E1, RC-III: Rice Cluster III, MB: *Methanobacteriales*, *Crenarchaea* Group 1.1C and Group 1.3b. The addition of 1 and 2 to MS, RC-I and RC-III labels represent sub-clustering at 97% sequence identity within each group.

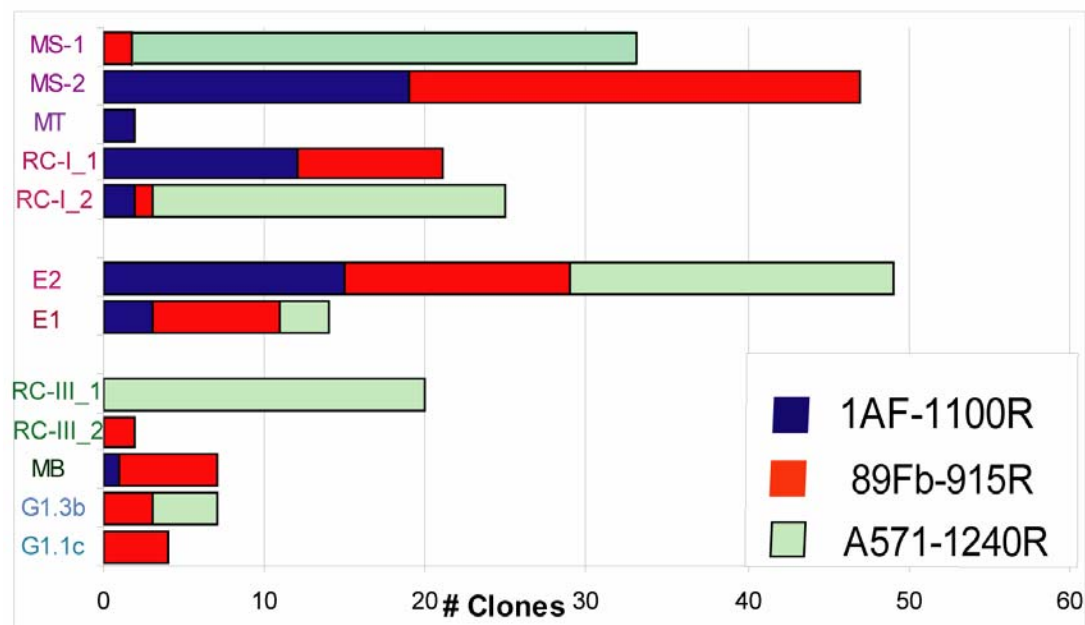


Figure A-5. Archaeal groups and their clone number as detected by each primer set used in this study. Sequences from *Dulichium* and *Sarracenia* samples were combined and distributed by each primer set. MS: *Methanosarcinaceae*, MT: *Methanosaetaceae*, RC-I: Rice cluster I, E2: group E2/Methanoregula, E1: group E1, RC-III: rice cluster III, and MB: *Methanobacteriales*.

Rarefaction analysis results showed that the community coverage started to plateau before all samples were taken for all the primers used in this study (Fig. A-6). Among the primers, the 89Fb-915R set had the most similar curve to the one for all primers at 97% sequence identity, followed by 1A-1100R and A751-1204. At 97% sequence identity, the A751-1204 rarefaction plot was closest to reaching saturation suggesting lower coverage for this primer. Similar trends were observed at 99% identity with a higher number of groups and a more exponentially shaped rarefaction curve, indicating that saturation was not close to being reached.

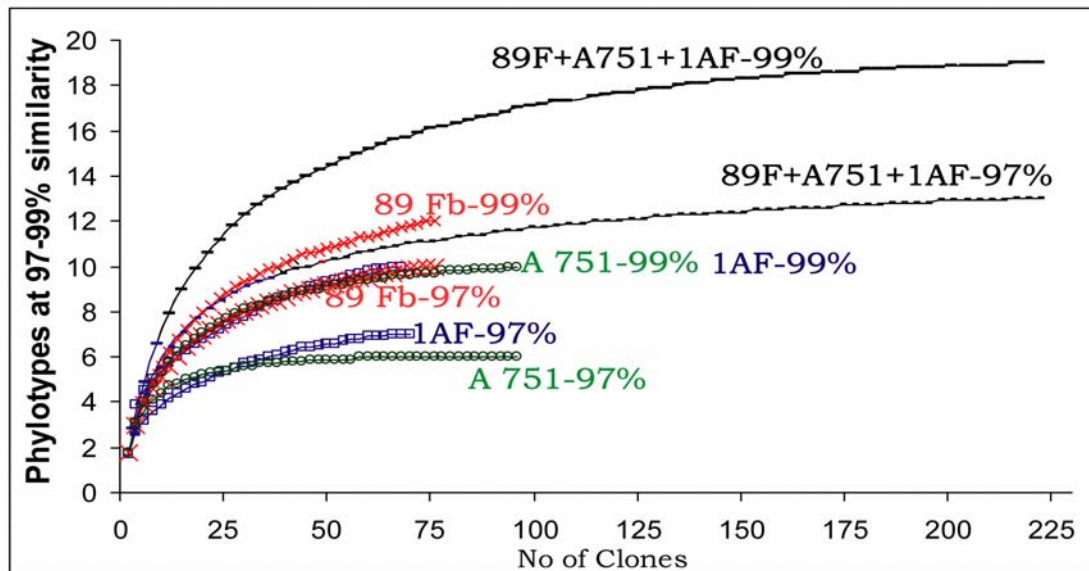


Figure A-6. Rarefaction analysis at 97 and 99% similarity levels for 16S rRNA gene sequences from combined *Dulichium* and *Sarracenia* samples for each primer set.

T-RFLP community analysis

T-RFLP analysis done with the 1AF-1100R primer set showed that the enzyme *Sau*96I produced more terminal-restriction fragments (T-RFs) from our samples than did similar attempts with the other two primers sets examined (not shown). In 1AF-1100R traces, some T-RFs were identified as corresponding to a unique phylogenetic group such as MS or RC-I, and several others were related to two or more groups.

In general, T-RFLP profiles indicated that the MS, RC-I and E2 groups made up the greatest proportion of the archaeal community in the rhizoplane of both plants (Fig. A-7A), and MS was a more significant fraction in *Sarracenia* than *Dulichium*. Additionally, the principal component analysis (PCA) of T-RFLP traces transformed into a presence/absence matrix showed that in general *Dulichium* samples cluster more closely together than *Sarracenia*. Interestingly, the *Sarracenia* samples were more similar to each other when separated by depth. ‘Upper’ and ‘lower’ sections (0-5 and

11-25 cm deep) formed cohesive clusters at different ends of the PCA plane, while the ‘middle’ sections (5-10 cm deep) formed a disperse group of points in between (Fig. A-7B). PCA differentiated *Dulichium* samples from ‘upper’ and ‘lower’ but not ‘middle’ samples of *Sarracenia* roots separated by depth (Fig. A-7B).

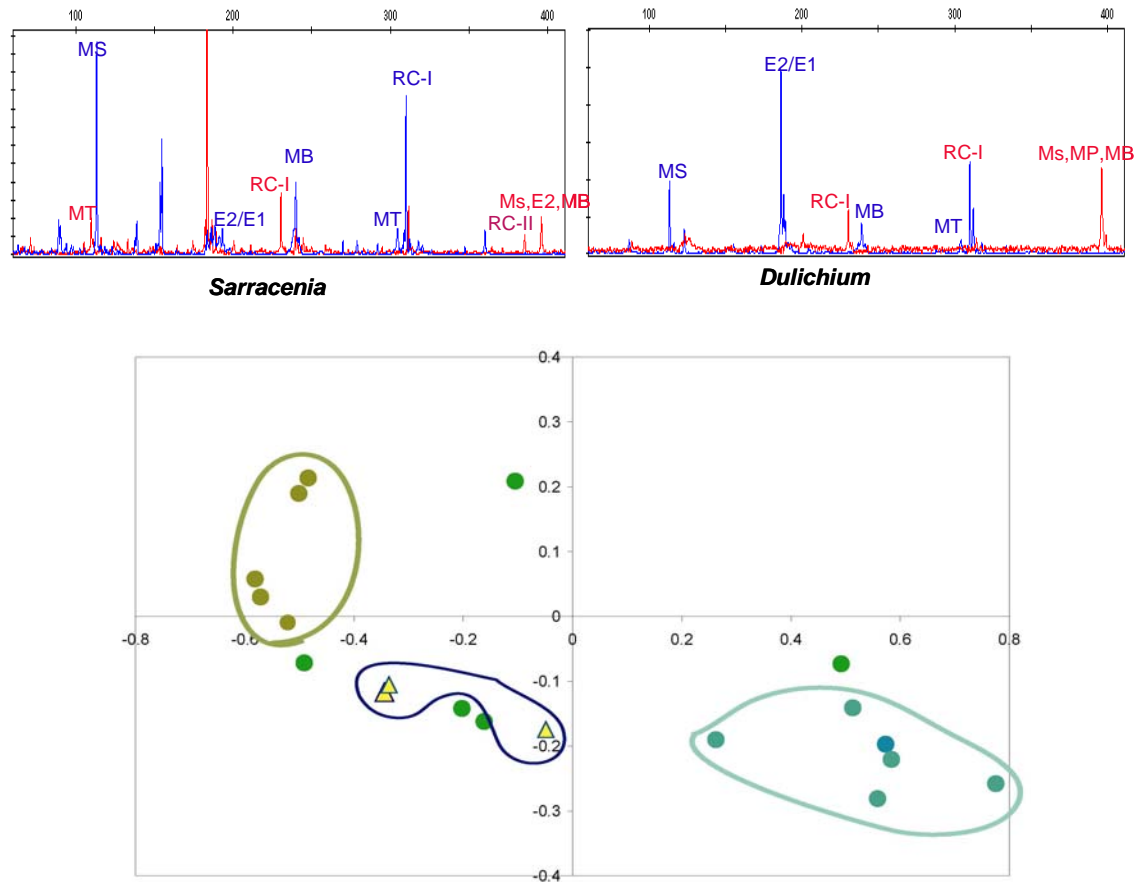


Figure A-7. A) Representative T-RFLP traces of 16S rRNA gene amplified with the 1AF-1100R primers labeled with 6-FAM (blue channel) and PET (red channel), respectively. PCR products were digested with *Sau96I* and peak identity was predicted from *in silico* digestion of clone libraries. MS: *Methanosarcinaceae*, MT: *Methanosaetaceae*, RC-I: Rice Cluster I, E2: group E2/Methanoregula, E1: group E1, and MB: *Methanobacteriales*. B) Principal component analysis (PCA) of T-RFLP traces after being transformed into a presence/absence matrix. *Sarracenia* samples (circles) were separated by depth: upper (light blue), middle (green) and lower (light brown), while *Dulichium* were not (triangles)

A.5 DISCUSSION

Previous 16S rRNA environmental surveys of the archaeal community in bulk peat from several acidic bogs found a similar euryarchaeal composition where groups such as MS, MT, RC-I, E2/*Methanoregula* and E1 were commonly present (Cadillo-Quiroz et al., 2006, Galand et al., 2003). However, the structure of the archaeal communities, as exemplified by 16S rRNA gene clone libraries, inhabiting the rhizoplane of both *Dulichium* and *Sarracenia* (Fig. A-3) were quite different from the previous observations in bulk peat soil from the same study site (Basiliko et al., 2003, Cadillo-Quiroz et al., 2006) or other acidic bogs (Galand et al., 2003, Kotsyurbenko et al., 2004, Rooney-Varga et al., 2007). The root system of *Dulichium spp.* (three-way sedge) and *Sarracenia purpurea* (pitcher plant) are located in the shallow peat layers of McLean Bog (5-30 cm from the surface), and our previous report showed that the bulk peat archaeal community in similar depths (10-20 cm) was uneven and dominated by the E2/'Methanoregula' group (Cadillo-Quiroz et al., 2006). The archaeal community in the rhizoplane in both plants did not show a single dominant group member, but instead 3 to 4 groups with close proportions (Fig. A-3), suggesting that *Dulichium* and *Sarracenia* roots exert influences leading to a more even archaeal community in McLean Bog. Many studies have shown that roots influence their surrounding microbial communities (Kent & Triplett, 2002), however their influences over community diversity (richness and evenness) have not been systematically addressed.

MS, RC-I and E2/'Methanoregula' were the co-dominant members in the rhizoplane of both plants. *Methanosarcina* (MS) is a metabolically versatile methanogen capable of using acetate, methylated compounds and H₂/CO₂ in some cases (Boone et al., 1993). The significant contribution of MS in the rhizoplane of both plants is supported by its metabolic capabilities to use several byproducts from

the metabolism of root exudates or decaying roots (Marschner & Römheld, 1996), its capability to tolerate low oxygen levels by different morphological and physiological mechanisms (Galagan et al., 2002, Zinder, 1993), and the reported occurrence of significant levels of acetate near rice roots (van Bodegom et al., 2001). MS made up for significant fraction of the rhizoplane community in both plants with 24 and 38% of clones in *Dulichium* and *Sarracenia*. The difference in MS contribution between the two plants (14%) was mainly accounted for by the contribution of MS-2 in *Sarracenia* samples. Unfortunately neither MS-1 nor MS-2 was closely related to an isolated MS strain (Fig. A-2), which would have enabled us to formulate a sound explanation for the differential contribution of MS-2. More culturing efforts are certainly required.

Methanosaeta (MT) is the only other reported acetate utilizing methanogen, and because it has a lower threshold for acetate use than MS (5 μ M versus 0.2 -1.2 mM respectively) (Jetten et al., 1992). It has been considered to be more abundant and apparently out competing MS in oligotrophic environments such as bogs and fens (Cadillo-Quiroz et al., 2008, Galand et al., 2005). However, MT represented a very small fraction in the roots of either plant. The concentration of oxygen and methanogenic nutrients (such as acetate, H₂, or methyl compounds) in the rhizoplane could play a significant role affecting the contribution of MT and several other groups to the archaeal community.

RC-I represented 20-21% of the clone libraries for the rhizoplanes of both plants. RC-I has been commonly found in the rhizosphere of rice plants (Grobkopf et al., 1998b) where it has been suggested to perform CH₄ production near roots driven by plant photosynthesis (Conrad et al., 2006). RC-I is a H₂/CO₂ utilizing methanogen as shown by a recent isolate (Sakai et al., 2007), which has also been detected (Cadillo-Quiroz et al., 2006, Galand et al., 2002) and enriched from peat samples (Sizova et al., 2003). RC-I has been detected as minor fraction in bulk peat of acidic

bogs (Cadillo-Quiroz et al., 2006). However, in sites with high abundance of vascular plants such as rice paddies or minerotrophic fen, it has been found to make up 20 to 50% of total methanogens (Kruger et al., 2005) or 5-14% of euryarchaeal clone libraries (Cadillo-Quiroz et al., 2008). It has been suggested that RC-I is well adapted to low H_2 habitats (Sakai et al., 2007) and relatively oxic conditions (Erkel et al., 2006), such as those in the rhizosphere and rhizoplane.

The group E2/*Methanoregula* is dominant in shallow bulk peat samples making up 55 to 90% of the total *Archaea* as estimated by quantitative PCR (Cadillo-Quiroz et al., 2006). A recent isolate (Bräuer et al., 2006) showed this group contains H_2/CO_2 utilizing methanogens with low optimal pH and highly sensitive to oxygen. These characteristics could represent a limiting factor for its contribution in the rhizoplane community. Plants have been found to alter the pH of the rhizospheric soils by more than two units when by excreting or reabsorbing H^+ or HCO_3^- , changing CO_2 concentrations by plant respiration, and releasing low molecular-weight root exudates (Marschner & Römheld, 1996). Given that the bulk peat in bogs has a low pH (3.5-4.0), then pH fluctuation to higher values is likely.

The E1 group represented a small fraction of the archaeal community in *Dulichium* (3%) and a slightly bigger fraction in *Sarracenia* (8%). This group has been found to be a minor fraction in shallow layers of the acidic McLean Bog and Chicago Bog but, at the same time, a more significant proportion of the community in deeper sedge-derived layers at Chicago Bog (Cadillo-Quiroz et al., 2006) and in a nearby neutral pH minerotrophic fen (Cadillo-Quiroz et al., 2008). This group has been shown to contain a H_2/CO_2 utilizing, mildly acidiphilic methanogen (Cadillo-Quiroz et al., 2008).

RC-III is a relative of the *Thermoplasmatales*, and in our clone libraries results the subcluster RC-III-1 displayed a large variation in its proportion in the rhizoplane of

either plant. *Dulichium* contained significantly more RC-III. RC-III is a diverse group previously detected in several terrestrial ecosystems such as rice roots and riparian soils (Grobkopf et al., 1998c, Kemnitz et al., 2004). Although an isolated representative has not yet been obtained, a member of this group grew in mixed enrichment from anoxic soils (Kemnitz et al., 2005). The characterization of the enrichment indicated that RC-III is a mesophilic, peptide-fermenting anaerobe with an optimal pH near 7.4 and no growth below pH 6.4 (Kemnitz et al., 2005). Root exudates and decomposition could provide RC-III with suitable conditions for heterotrophic growth and it is likely that the representatives in McLean Bog are more acidiphilic. Some minority groups such as MB and crenarchaeal group 1.3b (Jurgens et al., 1997) were present only in *Sarracenia* clone libraries while group 1.1c was presented in both plants. Similar crenarchaeal sequences have been observed as a major fraction of the archaeal community in grassland rhizosphere (Nicol et al., 2003), forest soils (Jurgens et al., 1997), and lakes (Jurgens et al., 2000).

In terms of 16S rRNA gene library coverage, the multiple primer approach allowed for a more detailed survey of archaeal groups. Rarefaction analysis at 97% sequence identity indicated the survey was close to reaching sampling saturation (Fig. A-6). None of the primer sets used in this study (Table A-1) recovered sequences from all archaeal groups (Fig. A-5). Surprisingly, the A571-UA1240R which was recently designed to target all *Archaea* (Baker et al., 2003), did not recover sequences of as many groups as the primers designed for mainly *Euryarchaea* (Hales et al., 1996) or *Crenarchaea* (Buckley et al., 1998). The 89Fb-915r had the broadest group coverage, while the 1AF-1100R was restricted to *Euryarchaea*. Based on these results we suggest a multi primer approach separately targeting either archaeal group than a single ‘universal’ set of primers.

Both primers of the 1AF-1100R set were fluorescently labeled for our T-RFLP analysis. No significant difference in amplification was observed when using both primers labeled (not shown), although different total fluorescence was observed in the same traces. This difference is explained by the difference in emission capacity from the fluorescent molecule as FAM has been reported to emit more fluorescence than PET (Applied Biosynthesis). We were able to match more peaks with a single archaeal group from the fragments generated from the reverse primer than the forward one. These results were used as the basis to define a methanogen specific T-RFLP with double enzyme digestion (Sau96I with HhaI) that we published elsewhere (Cadillo-Quiroz et al., 2006).

T-RFLP analysis supported our clone library findings that RC-I, MS and E2 were the dominant archaea on the rhizoplane of plants, and that MS was particularly abundant in *Sarracenia* (Fig. A-7A). Interestingly the PCA separation of *Sarracenia* samples by their depth reflects the physiological differences between the newly formed and expanding upper roots, the senescent deeper roots and the roots in between with mixed characteristics (Fig. A-1 and A-7B). *Sarracenia* is a perennial plant which is continually renewing its roots. Conversely, *Dulichium* showed a homogeneous clustering among samples from different points. This is not surprising given that the rhizome-stolon root system in this annual plant maintains its roots at similar depths and because of its secondary roots (but not the stolon) are renewed or become decayed in similar times along the growing or senescent season. This is not surprising given that *Dulichium* is an annual plant whose secondary roots are grown and recycled at similar times throughout the growing or senescent seasons.

In summary, our results indicated that although the rhizoplane of the evaluated vascular plants contain a similarly diverse archaeal community than bulk peat, the particular conditions of the rhizoplane have a strong influence on the community

composition which is markedly different than bulk soil. The ecological determinants in this habitat that favor the different archaeal groups deserve more attention.

A.6 ACKNOWLEDGEMENTS

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APPENDIX B
NOVEL *METHANOBACTERIUM* STRAINS FROM NORTHERN
TEMPERATE AND BOREAL PEATLANDS

B.1 ABSTRACT

Methanobacterium sp. strains, MH-SWAN1, SP6, AL-21 were isolated from north American peatlands and FIN-120 from a Finnish peatland. The cells of all four strains were rod-shaped, non-motile, stained Gram positive, and resisted lysis with 0.1% SDS. Cell sizes were 0.6 x 1.5-2.8, 0.65-0.7 x 2-3.4, 0.85 x 1.5-3, and 0.45 x 2.7-6.5 μm for strain MH-SWAN1, SP6, AL-21, and FIN-120, respectively, and all strains usually formed multicellular filaments in stationary phase. All the strains used H_2/CO_2 for methanogenesis, grew under mesophilic conditions (30°C), and grew under mildly acidic to near neutral pH (4.6-6.6). SP6 had the broadest pH range (<4.6 - >7.6), while MH-SWAN1 showed the narrowest range (4.8-6.6). The optimal pH for growth was 5.4-5.7, 5-5.4, 6.2 and 5.4-6.2 for MH-SWAN1, SP6, AL-21 and FIN-120, respectively. Maximal NaCl tolerance was 160 mM, >250 mM, 50 mM, and 250 mM for MH-SWAN1, SP6, AL-21, and FIN-120 respectively. Na_2S was toxic at very low concentrations for AL-21 (growth inhibition from 0.1 mM), while MH-SWAN1 and AL-21 were slightly less sensitive (inhibition at 0.8 mM sulfide), and SP6 did not show growth inhibition at concentrations up to 0.8 mM sulfide.

16S rRNA gene sequence analysis showed strain MH-SWAN1 had 95-97% identity with described *Methanobacterium* species. Strain SP6 was related to *M. bryantii*, *M. ivanovii* and *M. uliginosum* with 98% identity, forming an independent sister branch to these tree strains. SP6 sequence identity with the remaining *Methanobacterium* species was 94-96%. Strain AL-21 and FIN-120 were closely

related (99.5% identity), thus we consider them as members of the same species. AL-21 and FIN-120 clustered with environmental sequences and had 95-96% identity with other described *Methanobacterium*. We proposed these strains to be 3 new *Methanobacterium* species: *M. marshiensis* sp. nov. with SP6 as its type strain, *M. aciditolerans* sp. nov. with MH-SWAN1 as its type strain and *M. paludoborealis* with FIN-120 as type strain and AL-21 as a second strain.

B.2 INTRODUCTION

Peatlands cover around 5 million Km², contain nearly one third of all terrestrial carbon (Gorham, 1991), and are estimated to contribute around 20% of the total annual CH₄ emissions (Cicerone & Oremland, 1988, Harriss et al., 1985). Thus, peatlands and their inhabiting methanogenic *Archaea* are important in global carbon cycling and the atmospheric content of greenhouse gases. The major concentration of peatlands occurs in northern latitudes along the northern temperate, boreal, sub arctic and arctic regions (Gore, 1983, Gorham, 1991). Studies assessing the methanogenic *Archaea* from northern sites showed that in addition to new uncultured archaeal lineages, phylotypes associated with the orders *Methanomicrobiales*, *Methanosarcinales* and *Methanobacteriales* are the common inhabitants on these ecosystems (Basiliko et al., 2003, Galand et al., 2003, Kotsyurbenko et al., 2004, Metje & Frenzel, 2005). The importance of each group varies depending on the peatland type as observed in the comparisons of the archaeal community of minerotrophic fen versus bog (Cadillo-Quiroz et al., 2007, Cadillo-Quiroz et al., 2006). Most of the *Methanobacteriales* sequences recovered from peatlands are phylogenetically related to the genus *Methanobacterium*, and have been found in boreal (Kotsyurbenko et al., 2004) and temperate sites (Basiliko et al., 2003, Cadillo-

Quiroz et al., 2006). The net contribution of *Methanobacterium* spp. to peatland methanogenesis has not been assessed. However, recent *in vitro* manipulations of peat soil showed that lower pH conditions increased the presence of *Methanobacterium* as well as the contribution of hydrogenotrophic methanogenesis (Kotsyurbenko et al., 2007), suggesting a potential role of this group in some low pH environments. The *Methanobacterium* genus contains 8 formally accepted species in the latest edition of the Bergeys Manual of Systematic Bacteriology (Boone et al., 2001) in addition to 4 other proposed species (Cuzin et al., 2001, Joulain et al., 2000, Ma et al., 2005, Shlimon et al., 2004). The members in this group have been isolated from various sources (Boone et al., 1993) and present a broad range of characteristics, such as the G+C content (32-61%) (Boone et al., 2001).

Only two types of strains, *Methanobacterium palustris* and *Methanobacterium* sp. MB2- MB4, have been isolated from peatlands (Kotsyurbenko et al., 2007, Zellner et al., 1988). It is unlikely that these strains contain all the potential genetic and physiological diversity of this group in peatlands. Thus, more isolates are necessary to assess their properties and their potential role in peatlands. The present study describes the isolation and characterization of four novel *Methanobacterium* strains from temperate and boreal peatlands. We propose that these strains represent three novel *Methanobacterium* species.

B.3 METHODS

Anaerobically sampled peat soil was collected between June 2005 and September 2006 from the shallow layers (approximately 10-20 cm below surface) of four peatlands with the following locations and characteristics. Splinter Hill Marsh is forested by longleaf pine savanna/ seepage “bog” communities such as herbaceous

shrubs and *Sarracenia spp.*, and is located in Baldwin County southern Alabama, USA (30° 46'N, 87° 41'W). The site is a marshy wetland with sparse peat formation (5 to 10 cm deep) and acidic sandy soil (pH 4.5-5.2) and sparse peat accumulation (5-10 cm depth). Michigan Hollow is a minerotrophic temperate fen in upstate NY located near Danby NY, USA (42° 19' N, 76° 29'W), sedge-dominated site with near neutrality pH (~6.5). Baxter site (AL) is located near Anchorage Alaska (61° 32'N, 150° 27'W), and is an acidic bog with pH near 5.4 and dominated by *Sphagnum* mosses and ericaceous shrubs. AL samples were kindly provided by K. Duddleston. Lakkasuo mire complex is located in central Finland north of Orivesi (61° 47' N, 24° 18') (Laine et al., 2004), and is a commonly dominated by *Sphagnum* mosses. Samples were taken from the plot 10- eutrophic sedge fen, pH 5.1, as described by Laine et al. 2004. All samples were incubated as peat slurries supplemented with H₂/CO₂ and rifampicin as described by Brauer et al., (2006). Tubes that showed high methane accumulation in their headspace were selected for subsequent transfer into anaerobic medium peat medium 2 (PM2).

PM2 composition and preparation were as previously described (Cadillo-Quiroz et al., 2008) using the modified anaerobic technique of Hungate (Sowers & Noll, 1995). Several hours before inoculation, sterile anaerobic additions were made to the basal medium with the following final concentrations: 1.0 mM titanium (III) nitrilotriacetate (7.2 mL 1M Tris-HCl adjusted to pH 8, 4.8 mL of 0.5 M sodium nitrilotriacetate and 0.55 mL of 15% titanium III chloride from Riedel-de-Haen), 10 mM MES (2-(N-morpholino)) ethane sulfonic acid, pK_a = 6.2 at 28 °C, filter-sterilized 1 M stock solution adjusted to pH 7.8), 0.5 mM coenzyme-M (2-mercapthoethanesulfonic acid), 0.4 mM sodium acetate, 1% v/v vitamin solution (Balch et al., 1979) and 1 µmol H₂S (sterile gas). The final liquid volumes in the tubes

were ca. 5 ml, and 70.7 kPa H₂/CO₂ (80%/20%) was added to the headspaces unless otherwise specified.

Strains were obtained after several dilutions to extinction (10^{-8} or 10^{-9}) in PM2 medium adjusted to pH 5.6. The purity of the isolates was evaluated by microcopy, and growth under organic substrates such as yeast extract (0.2 g L⁻¹), glucose, or lactate (20 mM), all in the absence of rifampicin, to test for heterotrophic contaminants.

Cell morphology and motility were examined under phase contrast microscopy with a Nikon Eclipse E600 microscope equipped with a Hamamatsu CCD digital camera. Gram staining and susceptibility to SDS lysis were done as previously described (Boone & Whitman, 1988). SDS tests were done with the final concentrations ranging from 0.1% to 2%, and cell lysis was determined by microscopic observations. Negative staining transmission electron microscopy was done as described elsewhere (Firtel et al., 1995) using 2% uranyl acetate (pH 6.5) and a Phillips Technai 12 Biotwin electron microscope equipped with a Gatan Multiscan model 791 camera and Digital Micrograph software. Photos were taken at 100 KV.

For experiments on the effect of pH, the pH was adjusted by addition of 30 mM MES adjusted to various pH values. 15 mM citric acid additions were used to adjust pH values lower than 4.6 when needed. The pH values of the cultures were assessed at the end of incubations. For the Na₂S sensitivity test, Na₂S·9H₂O filter-sterilized anaerobic solutions were prepared with different concentrations so the same volume of reagent (0.05 mL to 5 mL cultures) was added to replicate cultures. To determine the effect of sodium on the growth of strain E1-9c^T, additions from sterile anaerobic NaCl stock solutions of different concentrations were made to achieve values up to 250 mM. The background sodium content, from the different components of the medium was ca. 15 mM. Treatments were monitored for CH₄ production with a

flame ionization gas detector as previously described (Cadillo-Quiroz et al., 2006). The cultures were incubated on a gyratory shaker at 30°C and 200 r.p.m and all evaluations were done for over 29 days.

16S rRNA gene amplification were done with the 1Af-1492r primers sets, using the same conditions as previously described (Cadillo-Quiroz et al., 2008, Hales et al., 1996). Phylogenetic analyses of 16S rRNA gene sequences were done with the ARB software (Ludwig et al., 2004) and the latest realease (January, 2007) of the “greengenes” database (DeSantis et al., 2006). The 16S rRNA gene sequences alignment was exported from ARB and phylogenetic trees were constructed by Bayesian analyses. The approximation of posterior probabilities was done with MrBayes version 3.0 (Ronquist & Huelsenbeck, 2003) using four-chain Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) analysis. Bayesian consensus trees were built with a burnout of 300 and posterior probabilities were calculated. Tree topology was confirmed using maximum likelihood and neighbor joining methods (implemented in ARB) with Olsen evolutionary distance correction.

B.4 RESULTS AND DISCUSSION

Cells from the four isolates had a rod-shaped morphology, with somewhat similar cell width but varying length (Fig. B-1). Cell sizes were 0.6 x 1.5-2.8, 0.65-0.7 x 2-3.4, 0.85 x 1.5-3, and 0.45 x 2.7-6.5 μm for strain MH-SWAN1, SP6, AL-21 and FIN-120, respectively. The occurrence of several cells forming filaments was commonly observed in SP6, AL-21 and Fin-120 cultures at stationary phase. Negative staining electron microscopy (Fig. B-1) allowed a more accurate cell size measurement, since phase contrast microscopy did not show enough resolution to distinguish between a long cell and a multicellular filament. Cells from all strains

stained Gram-positive and resisted lysis with 0.1% SDS, consistent with the presence of a pseudomurein containing cell wall, which is a unique characteristic of the *Methanomicrobiales* order (Sprott & Beveridge, 1993). Cells of all strains were non-motile which corresponded with the absence of flagella (Fig. B-1). However, fimbriae were observed in MH-SWAN1, AL-21 and Fin-120. Strain MH-SWAN1 showed a high abundance of fimbriae and its cell surface had a particularly coarse configuration (Fig. B-1).

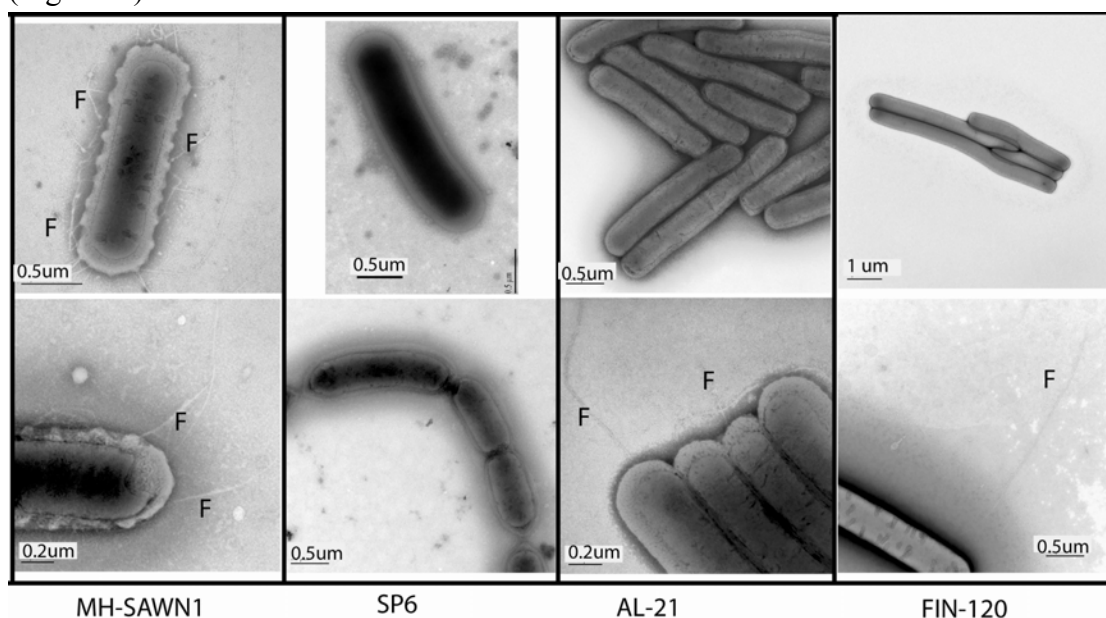


Figure B-1. Negative stain electron microscopy of strains MH-SWAN1, SP6, AL-21 and FIN-120. Magnification was adjusted to fit cell size or cell detail. Fimbriae are indicated as F.

All the strains were slightly acidiphilic, although with some differences in their pH optimum and range (Fig. B- 2). Strain MH-SWAN1 had a narrow pH range (4.8-6.6) with an optimum near 5.4-5.7. Strain SP6 had the broadest pH range (<4.6->7.6) with likely activity outside of our measured range. SP6's pH optimum was 5.0-5.4. Strain AL-21 had pH range from 4.7-7.1 with an optimum near 6.2. The pH range in FIN-120 was 4.7-7.6 and showed the broadest optimum (pH 5.4-6.2) of all the strains. The members of the *Methanobacterium* genus have been found to grow well near

neutrality with a few examples of alkaliphilic or acidiphilic members (Boone et al., 1993). *M. espanolae* was the only described acidiphilic member in the genus with the lowest pH range (4.6-7) and optimum (5.6-6.2) (Patel et al., 1990). Our isolated strains showed growth in a more acidic range and optimum pH. It is important to mention that a recent report indicated that a *Methanobacterium* strain MB4 grew down to pH 3.5 and had optimum pH at 5 (Kotsyurbenko et al., 2007); however we did not find a formal description of MB4 when writing this report. Outside of the Methanobacteriales order, only '*Candidatus Methanoregula boonei*', a member of the *Methanomicrobiales* order isolated from an acidic peatland (Bräuer et al., 2006), has similar or slightly lower acidiphilic values.

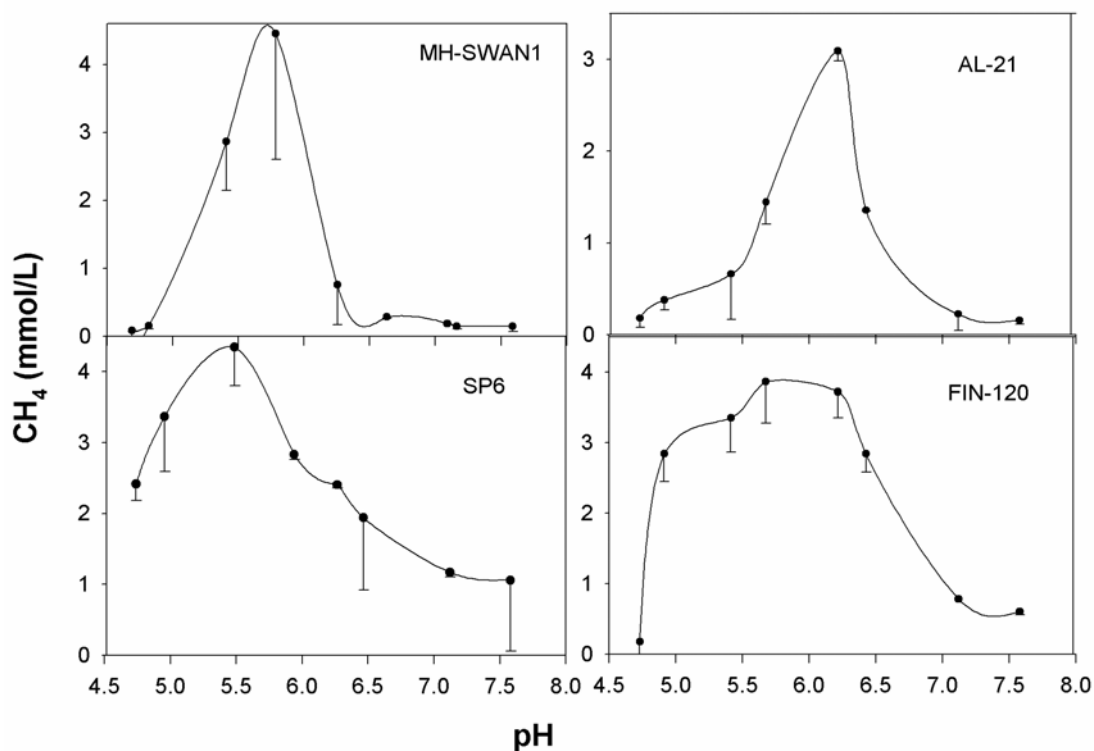


Figure B-2. Effects of pH on CH₄ production of strains MH-SWAN1, SP6, AL-21 and FIN-120. The y axis presents the accumulated CH₄ in culture headspace after 8 days of incubation at 30 °C under shaking conditions (220 r.p.m) with H₂/CO₂ as the methanogenic substrate.

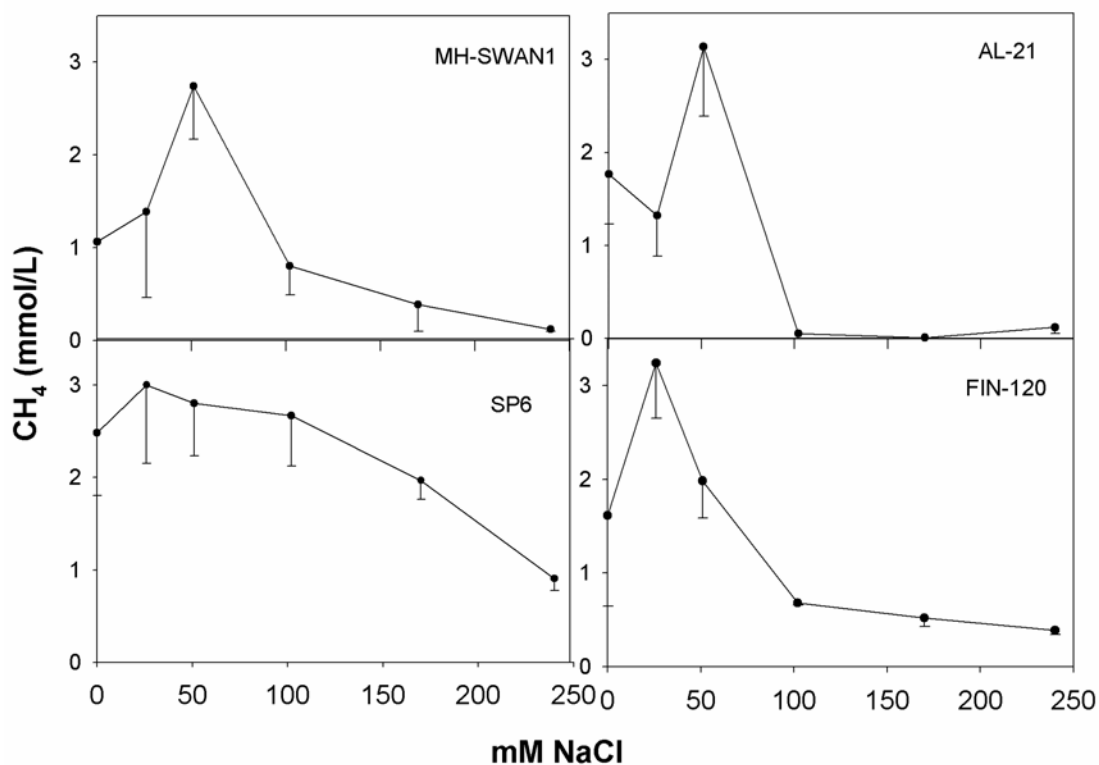


Figure B-3. Effects of NaCl on CH₄ production of strains MH-SWAN1, SP6, AL-21 and FIN-120. The y axis presents the accumulated CH₄ in culture headspace after 8 days of incubation at 30°C, pH 5.7, under shaking conditions (220 r.p.m) with H₂/CO₂ as the methanogenic substrate.

Low amounts of NaCl (10-60 mM) added to the ca. 15 mM Na⁺ already present in the medium slightly improved the growth over no NaCl addition. Concentrations above 160, 50, and 250 inhibited the growth of MH-SWAN1, AL-21 and FIN-120, respectively. SP6 was the most NaCl tolerant strain and showed significant growth up to 250 mM NaCl.

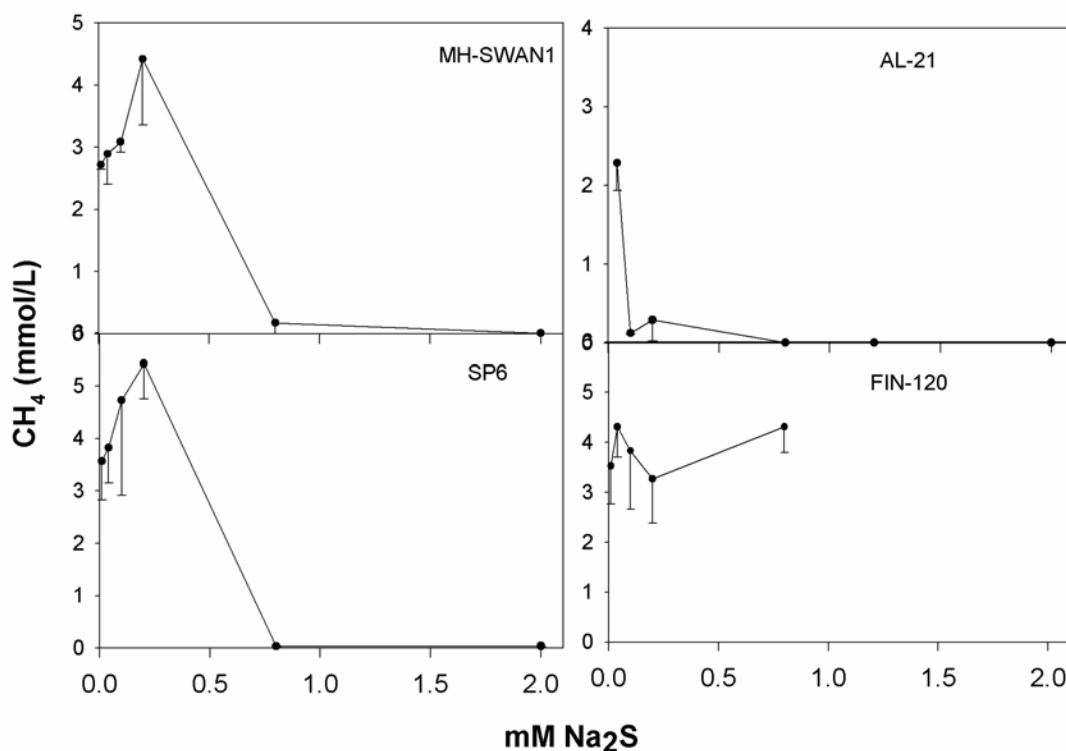


Figure B-4. Effects of Na₂S on CH₄ production of strains MH-SWAN1, SP6, AL-21 and FIN-120. The Y axis presents the accumulated CH₄ after 8 days of incubation at 30 °C, pH 5.7, under shaking conditions (220 r.p.m) with H₂/CO₂ as methanogenic substrate.

Strains AL-21 was highly susceptible to inhibition by Na₂S concentrations above 0.01 mM. Na₂S concentrations up to 0.4 mM improved the growth of strain MH-SWAN1 and SP6 but values above 0.4 mM inhibited their growth (Fig. B-4). Strain FIN-120 did not show growth inhibition with Na₂S concentrations up to 0.8 mM. Na₂S is used by methanogens as a sulfur source (Rajagopal & Daniels, 1986), is added as a reducing agent in growth media (Sowers & Noll, 1995), and has also shown concentration-dependant toxicity in anaerobic cultures (Brock & O'Dea, 1977, Mariotto et al., 1989). In agreement with our results, previous studies have shown a wide variability among methanogenic strains in terms of optimal and inhibitory Na₂S additions although most cultured strains are tolerant of Na₂S concentrations of 1 mM

or over typically used as reductants (Jarrell & Kalmokoff, 1988). Sensitivity to Na₂S has been observed in other isolates from peatlands (H. Cadillo-Quiroz, personal observation) and may limit the culturability of methanogens in sulfide-containing media.

The four strains grew under mesophilic conditions (30°C), and their metabolism is strictly anaerobic with H₂ used as the methanogenic substrate. In order to complete the physiological characterization of our *Methanobacterium* strains, tests for temperature effects and the use of other substrates such as formate, iso-propanol, iso-butanol and acetate, will be done in the near future.

In terms of 16S rRNA phylogenetic analysis, the four strains were found to be in the order *Methanobacteriales* within the genus *Methanobacterium* (Fig. B-5). Strain SP6 branched distantly from the other 3 isolates, and was associated with *M. bryantii*, *M. ivanovii* and *M. uliginosum* with 98% sequence identity to any of them. SP6 sequence identity to other more distant *Methanobacterium* species considered in our phylogenetic analysis was 94-96%. Strain MH-SWAN1 was closely related to *Methanobacterium* sp strain MB-4, *M. congolense* and *M. curvum* with 99%, 97% and 96% sequence identity. The sequence identity with other *Methanobacterium* species was between 95-96%. Strain MB4 was recently isolated from an acidic Siberian bog and suggested as a new species (Kotsyurbenko et al., 2007), although no formal description has been published up to this time.. Strain AL-21 and FIN-120 had very high sequence identity with each other (99.5%), and branched with two clones from a pH 4.5 H₂/CO₂ enrichment culture derived from a German acidic bog (Horn et al., 2003) with 98% sequence identity. Strain AL-21 and FIN-120 branched as a sister group to the cluster formed by *M. congolense*, strain MH-SWAN1 and MB4 (Fig. B-5) with 95-96% identity to them as well as the others *Methanobacterium* species considered in this analysis. The differences in sequence identity between the strains

described here and other species in this group was as low as or lower than among the already recognized species, suggesting they represent new species in the genus *Methanobacterium*.

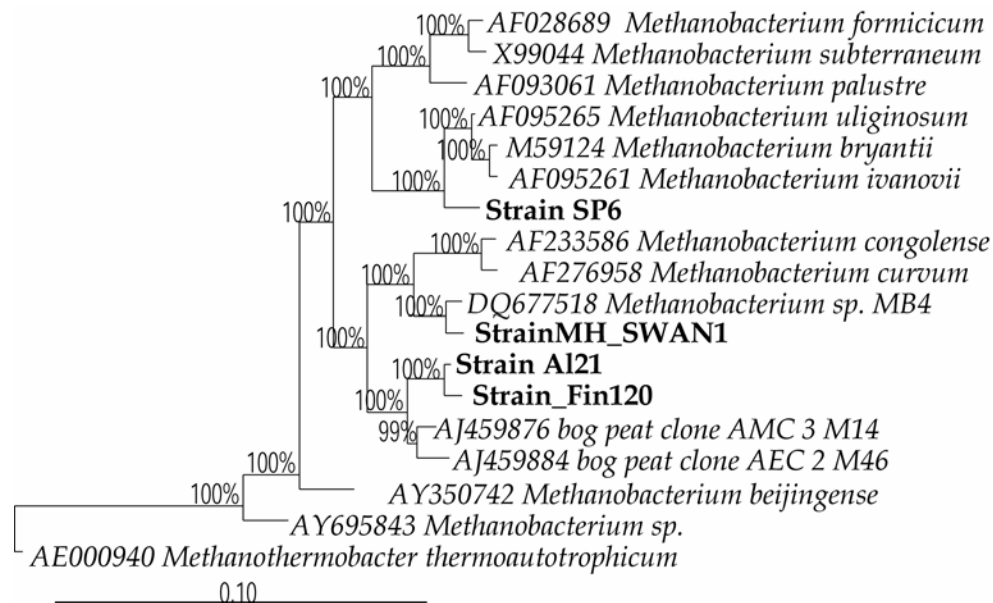


Figure B-5. 16S rRNA gene sequence phylogenetic analysis of strains MH-SWAN1, SP6, AL-21, FIN-120 and members of the order *Methanobacteriales*. Posterior probabilities above 98% are indicated in tree.

Considering the morphological, physiological (Table B-1) and phylogenetic characteristics, we propose that our isolated strains represent three novel *Methanobacterium* species. Preliminarily, we propose the following novel species with their respective type strains: *Methanobacterium marshiensis* sp. nov. with SP6 as its type strain, *Methanobacterium aciditolerans* sp. nov. with MH-SWAN1 as its type strain and *Methanobacterium paludoborealis* with FIN-120 as type strain and AL-21 as its second strain.

After completing a few additional physiological tests, a full taxonomic description for the proposed species will be included in the corresponding manuscript for taxonomic publication.

Table B-1. Physiological characteristics of novel strains versus other *Methanobacterium* species.

Organism	Source	Cell size	Substrates:		Growth temperature (°C)		pH for growth:		NaCl Range (M)	G+C content (mol%)	Na ₂ S range (mM)
			Formate	H ₂ , H ₂ S	Range	Optimum	Range	Optimum			
Strain MH-SWAN1 *	Microtrophic fen	0.6 x 1.3-2.8	ND	ND	ND	ND	4.8-6.6	5.4-5.7	0-0.17	ND	0-0.4
Strain SP6 *	Marshy soil	0.65-0.7 x 2-3.4	ND	ND	ND	ND	<4.6- >7.6	5-5.4	0- >0.24	ND	0-0.4
Strain AL-21 *	Bog	0.85 x 1.5-3	ND	ND	ND	ND	4.7-7.1	6.2	0-0.08	ND	0-0.01
Strain PIN-120 *	Bog	0.45 x 2.7-6.5	ND	ND	ND	ND	4.7-7.6	5.4-6.2	0-0.24	ND	0- >0.8
<i>Methanobacterium</i> sp. MB4 %	Bog	ND	+	ND	5-30	25-30	3.8-6	5-5.5	ND	ND	ND
<i>M. congluans</i> ^a	Anaerobic digester	0.4-0.5 x 2-10	- ^a	NG	25-30	37-42	5.9-8.2	7.2	ND	44.8 (Tm) ^a	ND
<i>M. pulvis</i> ^π	Peat bog	0.5 x 2.5-5	+	+	20-45	33-37	ND	7	0-0.3	34 (Tm)	ND
<i>M. nigrinosum</i> ⁶	Marshy soil	0.2-0.6 x 1.9-3.8	-	-	15-43	40	6-8.5	ND	ND	53.8 (Tm)	ND
<i>M. ivanovi</i> ⁰	Rock core	0.5-0.8 x 1-15	-	-	15-55	45	6.5-8.5	7-7.4	ND	36.6 (Tm)	ND
<i>M. bryantii</i> ^ε	Anaerobic digester	0.3-1 x 1.3-10	-	NG	ND	37-39	ND	6.0-7.2	ND	53-58 (Bd)	ND
<i>M. espanolae</i> ^ε	Sludge	0.8 x 3-22	-	NG	15-50	35	4.6-7.0	5.6-6.2	ND	34 (Tm)	ND
<i>M. oryzae</i> ^γ	Rice field	0.3-0.4 x 3-10	+	-	20-42	40	6.0-8.5	7	0-0.4	31 (T _e)	ND
<i>M. subterraneum</i> ^ρ	Deep granitic groundwater	0.1 x 0.6-1.2	-	-	3.6-43	20-40	6.3-9.2	7.8-8.8	0.2-1.3	54.5 (Tm)	ND
<i>M. alcaliphilum</i> ^φ	Alkaline lake	0.5-0.6 x 2-25	-	ND	ND	37	7.0-9.9	8.1-9.1	ND	57 (Bd)	ND

*: This study, %: Strain MB4 (Kotsyurbenko et al., 2007), ^a: strain DSM7095 (Cuzin et al., 2001), ^π: strain DSM 3108 (Zellner et al., 1988), ⁶: strain DSM 2956 (Konig, 1984), ⁰: strain DSM 2611 (Belyaev et al., 1986), ^ε: strain DSM 863 (Boone, 1987), ^ε: strain DSM OCM 178 (Patel et al., 1990), ^γ: strain DSM 11106 (Joulain et al., 2000), ^ρ: strain DSM 11074 (Kotelnikova et al., 1998), and ^φ: strain DSM 3387 (Worakit et al., 1986).

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